Investigating the regulation of the human decapping enzyme Dcp2

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Investigating the regulation of the human decapping enzyme Dcp2.

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

by

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2012
The dissertation of Stacy L Erickson is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

University of California, San Diego

2012
Dedication

This thesis is dedicated to my parents, my biggest supporters and loudest cheerleaders, who have always encouraged ambitious dreams and adventures, and who continue to show me that pursuing every endeavor with unwavering perseverance is the best way to live your life.
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Chapter 2, in full, is a reprint of the material as it appears in Erickson SL and Lykke-Andersen J. Cytoplasmic mRNP granules at a glance. Journal of
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Chapter 3, in full, is currently being prepared for submission for publication of the material as Erickson SL, Corpuz EO, Maloy JP, Fillman C, and Lykke-Andersen J. Hedls enhances decapping enzyme Dcp2 stability and cellular activity by interacting with a novel autoregulatory domain. The dissertation author is the primary investigator and author of this paper.
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ABSTRACT OF THE DISSERTATION

Investigating the regulation of the human decapping enzyme Dcp2.

by

Stacy L Erickson

Doctor of Philosophy in Biology

University of California, San Diego, 2012

Professor Jens Lykke-Andersen, Chair

Decapping is a critical step in several mRNA decay pathways that are important for the regulation of gene expression. A major decapping complex consists of the decapping enzyme Dcp2 as well as several decapping enhancers. Although several interactions between decapping factors have been mapped, little is known about how Dcp2 activity is controlled to target the appropriate mRNAs for decapping or how these enhancers regulate this activity. Through a deletion analysis, I identified an important autoregulatory domain that is responsible for Dcp2 protein turnover. I demonstrated that the metazoan specific decapping enhancer, Hedls, modulates Dcp2 stability and requires the autoregulatory domain of Dcp2 for interaction. Furthermore, Hedls
depletion reduces Dcp2 cellular activity independent of protein stability. Thus Hedls promotes Dcp2 mediated decapping by at least two mechanisms: controlling Dcp2 stability and enhancing Dcp2 catalytic activity. This could provide a way to modulate Dcp2 activity according to cellular demand, thereby preventing decapping of inappropriate targets.

Hedls is required for Dcp2 concentration in mRNP granules called processing bodies (PBs), which contain mRNA substrates targeted for decay and the associated mRNA decay factors. Because Hedls also modulates Dcp2 stability, I hypothesized that Dcp2 might be stabilized when associated with mRNA substrates as a mechanism for controlling Dcp2 levels according to cellular substrate levels. To test this, PBs were used as a tool to determine whether Dcp2 stability increases when concentrated with mRNPs. Through a deletion analysis, I found that the C-terminus of Hedls is required for concentration in PBs. Nevertheless, its deletion did not affect Dcp2 association or stabilization. I also examined a catalytically inactive Dcp2 mutant predicted to trap Dcp2 with mRNPs, but I did not find an increase in stability. Finally, I co-transfected an mRNA reporter containing a stem loop from Rrp41 mRNA for which Dcp2 has a high affinity and did not find stabilization. Therefore, exactly what dictates Dcp2 stability remains unknown and is an important topic for further investigation. In sum, my studies revealed an unexpected mechanism of Dcp2 regulation that could serve to prevent promiscuous Dcp2 activity thereby ensuring decapping of only mRNAs targeted for decay.
mRNA decay is a key step in the regulation of gene expression

Controlling the amount, timing, and location of the expression of genes into functional products is critical for essentially every process required for life, from organismal development to rapid response to stimuli. The process of turning on protein expression includes transcribing the gene into messenger RNA, which is used as a template for translation into protein. Once a gene is turned on it is often just as important to tightly regulate when it is turned off. While mRNA degradation is significantly less understood than other regulatory processes, it is a rapid and often used mechanism for silencing gene expression. In addition, mRNAs that have mistakes also need to be removed from the cell to prevent the production of truncated or mutant proteins. Therefore, mRNA decay is a powerful control mechanism, which is further illustrated by the association of mis-regulated mRNA decay with several human diseases such as cancer, inflammatory disease and Alzheimer’s disease (Hollams et al., 2002).

Eukaryotic cells have evolved extra protection for their mRNAs including the addition of a 5’ 7-methyl-guanosine (m$^7$G) cap to the newly synthesized 5’ end and polyadenylation of the 3’ end of most mRNAs after transcription termination. This protects the RNA from degradation from either end by exonucleases. In addition, the m$^7$G cap is required for the initiation of
translation of the majority of mRNAs. Therefore, regulating the removal of the m\(^7\)G cap is a key step in silencing gene expression as it simultaneously shuts down translation initiation and activates decay of the message (Coller and Parker, 2004; Eulalio et al., 2007a; Franks and Lykke-Andersen, 2008; Simon et al., 2006).

Catalysis of decapping by Dcp2 and associated factors

The central components of the decapping complex, which are conserved from yeast to humans, include the catalytic subunit Dcp2 and its co-activator Dcp1. The phosphohydrolase activity of Dcp2 requires an N-terminal Nudix (Nucleoside Diphosphate linked to X) motif to release m\(^7\)Gpp from the mRNA body (Dunckley and Parker, 1999; Lykke-Andersen, 2002; van Dijk et al., 2002; Wang et al., 2002). Dcp2 requires RNA for cap recognition and decapping efficiency increases with longer substrates (Piccirillo et al., 2003; van Dijk et al., 2002). Residues within the Nudix domain form an RNA binding channel, which contacts RNA through weak non-specific interactions and also makes specific contacts with the cap (Deshmukh et al., 2008). An additional Nudix family member called Nudt16 or X29 was recently shown to have cytoplasmic activity in mammals, although little else is known about this enzyme (Song et al., 2010).

As illustrated in Figure 1.1, many proteins that associate with Dcp2-Dcp1 and enhance decapping have been identified, including the RNA
Figure 1.1: The decapping enzyme Dcp2 and associated factors. Several interactions between the proteins that activate 5’ to 3’ decay have been mapped in various organisms. Competing interactions have also been identified and thus it is currently unclear how many different versions of the decapping complex exist in various organisms. This schematic is a representation of the generally conserved interactions known thus far. (a) The decapping complex (indicated by the top bracket), as identified by co-immunoprecipitation experiments in human cells, is comprised of Dcp2, Dcp1, Hedls, Edc3, and Rck/p54 (Fenger-Grøn et al., 2005). These proteins associate in other organisms as well, but the specific interactions can vary. For example, Hedls is required for the Dcp1:Dcp2 interaction in animals and the interaction of Edc3 with Dcp1 and/or Dcp2 varies depending on the species (Fenger-Grøn et al., 2005; Nissan et al., 2010; Ozgur et al., 2010; Tritschler et al., 2007). (b) The translational repressors (indicated by the middle bracket) include Rck/p54, Scd6, and Lsm-Pat1. These factors can interact with each other and can compete, as indicated by studies in yeast and flies, for Dcp1 and/or Dcp2 binding as well as association with other factors in the decapping complex (Fromm et al., 2012; Haas et al., 2010; Nissan et al., 2010; Ozgur et al., 2010). As a result of translation inhibition, these factors enhance decapping in cells, but only Pat1 and Scd6 directly enhance yeast Dcp2 activity in vitro. (c) The 5’ exonuclease Xrn1 (indicated by the bottom bracket) was also found to associate with Pat1 in yeast and human cells but not in flies (Haas et al., 2010; Nissan et al., 2010; Ozgur et al., 2010).
helicase Rck/p54 (Decker et al., 2007; Lykke-Andersen and Wagner, 2005; Nissan et al., 2010), the Lsm-Pat1 complex (Haas et al., 2010; Nissan et al., 2010; Ozgur et al., 2010), Scd6 (Fromm et al., 2012; Nissan et al., 2010), and the enhancer of decapping (Edc) proteins, Edc3 (Decker et al., 2007; Fenger-Grøn et al., 2005; Nissan et al., 2010; Tritschler et al., 2007) and metazoan-specific Hedls (Fenger-Grøn et al., 2005; Xu et al., 2006; Yu et al., 2005). Two additional enhancers of decapping, Edc1 and Edc2, have been identified in S. cerevisiae (Schwartz et al., 2003; Steiger et al., 2003). In addition, the 5’ to 3’ exonuclease Xrn1 interacts with the Lsm-Pat1 complex (Bonnerot et al., 2000; Bouveret et al., 2000; Nissan et al., 2010; Ozgur et al., 2010). Table 1.1 lists orthologs for these proteins in organisms where mRNA decay pathways have been studied. It is currently unclear to what extent these factors are recruited to mRNPs as one large complex or as smaller sub-complexes; however, some interactions between decapping effectors appear to be mutually exclusive, suggesting dynamic or alternative complex formation (Decker et al., 2007; Fromm et al., 2012; Haas et al., 2010; Pilkington and Parker, 2008; Tritschler et al., 2009; Tritschler et al., 2008).

Although several decapping enhancers have been identified, the mechanisms by which they act to enhance Dcp2-mediated decapping of mRNAs targeted for decay are largely unknown. The most studied regulator is Dcp1, which is required for decapping in yeast in vivo (Beelman et al., 1996; Sakuno et al., 2004; Steiger et al., 2003). Structural studies in yeasts show
Table 1.1: Orthologs of factors involved in PB and SG formation. PB (processing body) and SG (stress granule) components are conserved and concentrate in mRNP granules from yeast to humans. Components that have been identified in granules are listed for each organism. (a) Putative orthologs identified by sequence similarity. (b) Arabidopsis XRN4 is a cytoplasmic homolog of human Xrn2. x: No obvious ortholog. y: Multiple possible orthologs. The indicated orthologs were identified in: Lsm 1-7 (Eulalio et al., 2007b; Ingelfinger et al., 2002; Schneider et al., 2006; Sheth and Parker, 2003); Pat1 (Eulalio et al., 2007b; Scheller et al., 2007; Sheth and Parker, 2003); Xrn1 (Bashkirov et al., 1997; Ingelfinger et al., 2002; Schneider et al., 2006; Sheth and Parker, 2003; Weber et al., 2008); Dcp1/Dcp2 (Behm-Ansmant et al., 2006; Ingelfinger et al., 2002; Iwasaki et al., 2007; Lall et al., 2005; Sheth and Parker, 2003; van Dijk et al., 2002; Xu et al., 2006); Hedls (Eulalio et al., 2007b; Fenger-Grøn et al., 2005; Xu et al., 2006; Yu et al., 2005); Edc3 (Eulalio et al., 2007b; Fenger-Grøn et al., 2005; Kshirsagar and Parker, 2004); Rck/p54 (Behm-Ansmant et al., 2006; Cougot et al., 2004a; Lall et al., 2005; Sheth and Parker, 2003; Xu et al., 2006); Rap55 (Audhya et al., 2005; Boag et al., 2005; Decker and Parker, 2006; Eulalio et al., 2007b; Xu and Chua, 2009; Yang et al., 2006); eIF4E-T (Andrei et al., 2005; Ferraiuolo et al., 2005; Wilhelm et al., 2005); TIA-1/R (Buchan et al., 2008; Kedersha et al., 1999; Weber et al., 2008); and G3BP (Tourrière et al., 2003).
<table>
<thead>
<tr>
<th>Lsm-Pat1 complex</th>
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<th><em>Drosophila melanogaster</em></th>
<th><em>Caenorhabditis elegans</em></th>
<th><em>Arabidopsis thaliana</em></th>
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</tr>
</thead>
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<td>Lsm1-7</td>
<td>LSM-1-7</td>
<td>Lsm1-7</td>
<td>Lsm1-7p</td>
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<tr>
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<td>PatL1</td>
<td>HPat</td>
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</tr>
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<td>DCP1, 2</td>
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</tr>
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<td>[C18A3, Y46G5A] (a)</td>
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that Dcp1 promotes conformational changes in Dcp2 that might regulate its activity (Deshmukh et al., 2008; Floor et al., 2010; She et al., 2008). Curiously though, several pieces of data suggest that the exact mechanism by which Dcp1 enhances Dcp2 activity in yeast is not conserved. For example, Dcp1 does not directly interact with Dcp2 in human cells or enhance the *in vitro* activity of Dcp2 in human cells, *C. elegans*, and *D. melanogaster* (Cohen et al., 2005; Fenger-Grøn et al., 2005; Iwasaki et al., 2007; Lin et al., 2008; Lykke-Andersen, 2002; van Dijk et al., 2002).

Interestingly, a metazoan-specific decapping enhancer called Hedls might explain these differences. We previously identified Hedls in human cells as part of a larger decapping complex that facilitates the interaction between Dcp2 with Dcp1 and additional interactions with Edc3 and Rck/p54. Hedls purified from extracts enhances Dcp2 activity in vitro implicating Hedls as an enhancer, but the mechanism of enhancement was not understood (Fenger-Grøn et al., 2005). Studies of Dcp2 from *A. thaliana* showed that bacterially expressed Hedls and Dcp1 can directly interact with and enhance Dcp2 activity in vitro demonstrating that Hedls is an enhancer but also that Dcp1 can directly enhance decapping (Xu et al., 2006). Therefore, the mechanism by which Hedls enhances Dcp2 in other metazoans including human cells may be different and is addressed in research presented in this thesis.

**Activation of decapping in general mRNA decay**
While less is understood about the relative contributions of decay from the 5’ or 3’ ends in human cells, studies in *S. cerevisiae* suggest how the bulk of mRNA decay proceeds. The initiation of decay typically begins with deadenylation of the poly(A) tail performed by a variety of deadenylase complexes (Coller and Parker, 2004). Subsequently, decapping is triggered from the 5’ end, which is catalyzed by the enzyme Dcp2 (Dunckley and Parker, 1999). This allows for degradation by the 5’ to 3’ exonuclease Xrn1 (Decker and Parker, 1993; Hsu and Stevens, 1993). Alternatively, decay can also occur from the 3’ end by a multienzyme complex called the exosome (Liu et al., 2006).

How is decapping activated by 3’ deadenylation? A complex of Lsm proteins, which form a heptameric ring structure similar to the Sm complex involved in splicing, has an inherent affinity for deadenylated transcripts and protects the transcript from 3’ decay (Chowdhury et al., 2007; He and Parker, 2001; Tharun and Parker, 2001). Lsm1-7 associates with another protein called Pat1, which represses translation initiation in addition to activating decay through interactions with both the decapping machinery and deadenylases (Haas et al., 2010; Nissan et al., 2010; Ozgur et al., 2010; Pilkington and Parker, 2008). This suggests that the Lsm-Pat1 complex bridges deadenylation with 5’ decay by recruiting the decapping machinery and preventing further 3’ decay.
Specialized mRNA decay pathways that trigger decapping

Although Dcp2 can non-specifically bind RNA *in vitro*, decapping complexes are often recruited to mRNPs either through their intrinsic affinity for specific RNA sequences or structures or through recruitment by mRNA-specific factors. For example, Dcp2 and Edc3 can interact directly with specific target mRNAs (Badis et al., 2004; Dong et al., 2007; Li et al., 2008) and the Lsm-Pat1 complex binds and directs the recruitment of the decapping machinery to deadenylated mRNAs as mentioned above (Chowdhury et al., 2007; Haas et al., 2010; Tharun et al., 2000; Tharun and Parker, 2001).

Several mRNA-specific factors have also been identified that bind cis-elements in the mRNA and recruit the decapping complex to activate mRNA decay. One such factor is the RNA-induced silencing complex (RISC), which targets mRNAs for silencing by its associated micro (mi)RNAs or small interfering (si)RNAs. Depending on the specific interaction between the miRNA and mRNA, RISC appears to induce mRNA silencing by different mechanisms, including translational repression, deadenylation, and decapping, or, in the case of near-perfect miRNA-mRNA base pairing, endonucleolytic cleavage (Eulalio et al., 2008; Fabian et al., 2010; Wu and Belasco, 2008). Although the extent to which targets rely on decapping is unclear and silencing mechanisms have been controversial, the decapping complex has been shown to be important for silencing in several studies in various organisms (Behm-Ansmant et al., 2006; Eulalio et al., 2007b; Li et al., 2011; Rehwinkel et al., 2005).
Other factors that promote mRNA-specific decapping complex recruitment are the proteins TTP and BRF-1, which bind AU-rich elements (AREs) found in the 3’ untranslated region (UTR) of many unstable mRNAs. These proteins activate the decay of ARE-containing mRNAs by recruiting mRNA decay factors, including deadenylases and decapping factors. Interestingly, the relative levels of activation of 5’ and 3’ decay varies depending on the specific ARE (Chen et al., 2001; Fenger-Grøn et al., 2005; Gao et al., 2001; Lykke-Andersen and Wagner, 2005; Mukherjee et al., 2002; Murray and Schoenberg, 2007; Stoecklin et al., 2006). In addition, a recent study identified ARE-containing mRNAs that are differentially targeted for decapping by either or both of the two cytoplasmic decapping enzymes, Dcp2 and Nudt16 (Li et al., 2011). Finally, TTP also interacts with the RISC component Ago2 (Jing et al., 2005), which might further facilitate decapping machinery recruitment through the RISC complex.

The Upf proteins, which are involved in targeting mRNAs with premature termination codons for nonsense-mediated decay, are also known to associate with the decapping complex (Cho et al., 2009; Fenger-Grøn et al., 2005; He and Jacobson, 1995; Lykke-Andersen, 2002). Although, the importance of decapping in this mRNA decay pathway varies between organisms (Muhlemann and Lykke-Andersen, 2010), the recent study on the two decapping enzymes in MEF cells, showed that Dcp2 contributed to decay
but was not essential for NMD, while Nudt16 did not play a role (Li et al., 2011).

**Localization of the decapping complex in mRNP granules**

One of the most curious aspects of the 5’ to 3’ decay machinery is that these factors, the mRNA-specific factors with which they associate, and their mRNA targets colocalize in dynamic cytoplasmic foci called processing bodies (PBs). These proteins include Dcp2 and the decapping enhancers mentioned previously, the 5’ to 3’ exonuclease Xrn1 (see Table 1.1), and the factors associated with miRNA-mediated decay (Jakymiw et al., 2005; Liu et al., 2005; Pauley et al., 2006), ARE-mediated decay (Franks and Lykke-Andersen, 2007), and NMD (Durand et al., 2007; Sheth and Parker, 2006). Several other proteins have also been identified, but the factors mentioned all have effects on PB dynamics, which is further discussed in the next chapter and in my results presented in this thesis.
Chapter 2. Cytoplasmic mRNP granules at a glance

Introduction

From their transcriptional birth to their degradation, cellular mRNAs are coated with proteins in messenger ribonucleoprotein (mRNP) complexes. The mRNP composition controls every aspect of the life of the mRNA, from pre-mRNA processing to mRNA localization, translation and turnover. Transitions between these events are accompanied by major mRNP remodeling and exchange of mRNP proteins. Upon entering the cytoplasm, the mRNP composition dictates whether the mRNA engages in translation, or remains translationally inactive and is subject to either storage or degradation (see Poster). In recent years, it has become clear that many translationally inactive mRNPs have the ability to assemble into cytoplasmic mRNP granules (for reviews see Anderson and Kedersha, 2009; Arkov and Ramos, 2010; Buchan and Parker, 2009; Eulalio et al., 2007a; Franks and Lykke-Andersen, 2008; Kulkarni et al., 2010; Zeitelhofer et al., 2008). The best-characterized mRNP granules in the somatic cell cytoplasm are processing bodies (PBs) and stress granules (SGs). To outline the current understanding of cytoplasmic mRNP granules, we will discuss the protein complexes required for the assembly of mRNPs into PBs and SGs, the conditions under which assembly occurs and the potential outcomes of assembling mRNPs into large macromolecular complexes. This discussion is relevant also to other cytoplasmic mRNP
**Figure 2.1 (Poster):** The cytoplasmic mRNP cycle, key mRNP granule assembly factors (human), and morphology of PBs and SGs. Abbreviations: 4E-T, eukaryotic translation initiation factor 4E transporter; 40S, small ribosomal subunit; 60S, large ribosomal subunit; AAAA, polyadenylate tail; CBC, nuclear cap binding complex; DAPI, 4’,6-diamidino-2-phenylindole; DCP1 and 2, decapping 1 and 2; DDX6, DEAD (asparagine-glutamine-alanine-asparagine) box polypeptide 6; EDC3 and 4, enhancer of decapping 3 and 4; eIFs, eukaryotic translation initiation factors; G3BP, RasGAP SH3-domain-binding protein; GW182, glycine-tryptophan protein of 182 kilodaltons; LSM1–7, Sm-like 1–7; m7G, 7-methylguanosine cap; MD, multimerization domain; mRNA, messenger ribonucleic acid; mRNP, messenger ribonucleic acid-associated protein; PABP, polyadenylate-binding protein; PATL1, protein associated with topoisomerase II homolog L1; PB, processing body; Q/N-rich, glutamine/asparagine-rich; RAP55, ribonucleic-acid-associated protein 55; RISC, ribonucleic-acid-induced silencing complex; SG, stress granule; TIA-1 and TIAR, thymus cell intracellular antigen 1 and TIA related; TTP, tristetraprolin; UPF1, upframeshift 1; XRN1, exoribonuclease 1.
Cytoplasmic mRNP Granules at a Glance
Stacy L. Erickson and Jens Lykke-Andersen

The cytoplasmic mRNP cycle

Key mRNP granule assembly factors (human)

<table>
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<tr>
<th>Factor</th>
<th>Function</th>
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<tr>
<td>Processing bodies</td>
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<tr>
<td>DCP2-DCP1</td>
<td>Decapping complex</td>
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<tr>
<td>XRN1</td>
<td>5’ to 3’ exoribonuclease</td>
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<td>EDC1, EDC4</td>
<td>Enhancers of decapping</td>
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<tr>
<td>DDMD, LSM1-7, PATL1</td>
<td>Translation initiation repressors and decapping enhancers</td>
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<tr>
<td>RAP55, 4E-B</td>
<td>Translation repressors</td>
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<tr>
<td>GW182</td>
<td>RISC component</td>
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<td>Stress granules</td>
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<td>TIA-1, TIAR</td>
<td>Translation repressors</td>
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<td>Q3BP</td>
<td>RhoGAP-associated endoribonuclease</td>
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Morphology of PBs and SGs

MelA cells, heat shocked at 42°C for 1 hour
granules, for example those found in germ cells and neuronal cells, and during early development (Anderson and Kedersha, 2009; Arkov and Ramos, 2010; Zeitelhofer et al., 2008), because these mRNP granules probably function in a similar manner to PBs and SGs.

**Morphology and movement of PBs and SGs**

PBs and SGs are highly dynamic membraneless cytoplasmic granules of translationally repressed mRNPs observed in a wide variety of eukaryotes. Whereas SGs are primarily observed during cell stress, PBs are generally observed under normal growth conditions, although in human cell lines, visible PBs disappear during mitosis and quiescence (Yang et al., 2004). Under the microscope, PBs generally seem discrete and rounded, whereas SGs can seem more diffuse (see Poster). Electron microscopy of human cells shows that PBs can range from 100 to 300 nm in diameter (Yang et al., 2004) and SGs, formed upon overexpression of the SG assembly factor TIA-1 (T-cell-restricted intracellular antigen 1), can average 100-200 nm (Gilks et al., 2004; Yang et al., 2004). Fluorescence recovery after photobleaching (FRAP) experiments revealed that many components cycle rapidly in and out of PBs and SGs, although a subset are more static (Aizer et al., 2008; Andrei et al., 2005; Eisinger-Mathason et al., 2008; Fujimura et al., 2008a, b; Guil et al., 2006; Kedersha et al., 2000; Kedersha et al., 2005; Leung et al., 2006; Mollet et al., 2008). Real-time imaging of human cell lines shows that most PBs and
SGs move in an apparently random manner. A subset of PBs can appear static, whereas occasionally rapid directional movement of PBs or SGs can be observed (Aizer et al., 2008; Kedersha et al., 2005; Nadezhdina et al., 2010; Yang et al., 2004). Some evidence indicates a role for the cytoskeleton in PB and SG dynamics. For example, microtubule-depolymerizing drugs can lead to impaired SG formation (Fujimura et al., 2009; Ivanov et al., 2003; Kolobova et al., 2009; Kwon et al., 2007; Loschi et al., 2009), impaired SG and PB movement and enlarged PBs (Aizer et al., 2008; Sweet et al., 2007). By contrast, actin depolymerization does not affect SG assembly (Ivanov et al., 2003; Kwon et al., 2007) and PBs associated with actin in human cells do not appear mobile (Aizer et al., 2008). Microtubule motor proteins can also affect SG and PB dynamics. Inhibition of dynein function can lead to impaired SG formation and enlarged PBs in response to stress (Kwon et al., 2007; Loschi et al., 2009; Tsai et al., 2009), whereas depletion of kinesins can delay the disassembly of SGs and rescue the assembly defects caused by dynein depletion (Loschi et al., 2009). Although these observations suggest functional interplay between SGs and PBs and the cytoskeleton, pleiotropic effects arising from cytoskeletal manipulation make it difficult to pinpoint its importance and relevance.

The composition and function of PBs

Factors involved in PB assembly
PBs are assemblies of translationally inactive mRNPs and RNA is central to the PB structure. Accordingly, PBs dissociate upon RNase treatment of permeabilized *Drosophila S2* cells and *Saccharomyces cerevisiae* cell extracts (Eulalio et al., 2007b; Teixeira et al., 2005). Ribosomal subunits have not been detected in PBs, suggesting that mRNPs must be free of ribosomes to assemble into a PB. This is further supported by evidence that trapping mRNPs in complex with ribosomes using translation elongation inhibitors prevents PB assembly (Cougot et al., 2004a; Eulalio et al., 2007b; Teixeira et al., 2005). Conversely, conditions that inhibit mRNP association with ribosomes can enhance PB assembly (Brengues et al., 2005; Cougot et al., 2004a; Eulalio et al., 2007b; Franks and Lykke-Andersen, 2007; Teixeira et al., 2005). Although the lack of associated ribosomes appears to be a precondition for the assembly of an mRNP into PBs, evidence suggests it is not sufficient; mRNPs also need to associate with protein complexes that promote PB assembly (Eulalio et al., 2007b; Franks and Lykke-Andersen, 2007).

Key factors that promote the assembly of mRNPs into PBs include decapping factors and associated proteins that repress translation initiation, and activate decapping and 5’ to 3’ decay of the mRNA (see Poster) (for reviews, see Coller and Parker, 2004; Eulalio et al., 2007a; Franks and Lykke-Andersen, 2008; Simon et al., 2006). Evidence suggests that multiple alternative complexes between these factors exist that, depending on the specific complex composition and on cell conditions, either promote the
repression of translation initiation only or additionally activate 5' to 3' decay of target mRNAs (Decker et al., 2007; Haas et al., 2010; Pilkington and Parker, 2008; Tritschler et al., 2009; Tritschler et al., 2008; Yoon et al., 2010). Several lines of evidence suggest that recruitment of these 5’ repression-decay complexes allows mRNP assembly into PBs (Anderson and Kedersha, 2009; Balagopal and Parker, 2009; Eulalio et al., 2007a; Franks and Lykke-Andersen, 2008; Kulkarni et al., 2010). First, all of these factors are enriched in PBs. Second, PB formation is impaired upon depletion of factors that recruit the 5’ repression-decay complexes to mRNAs (Eulalio et al., 2007b; Jakymiw et al., 2005; Liu et al., 2005; Pauley et al., 2006; Sheth and Parker, 2003; Zheng et al., 2008). Third, depletion or overexpression of individual 5’ repression-decay factors affects PB formation (Anderson and Kedersha, 2009; Balagopal and Parker, 2009; Eulalio et al., 2007a; Franks and Lykke-Andersen, 2008; Kulkarni et al., 2010). The specific effects that manipulating different 5’ repression-decay factors have on PBs can vary between organisms and conditions, probably reflecting differences in factors that are rate limiting for formation of the most abundant mRNPs in the PB under the given conditions.

**Recruitment of 5’ repression-decay complexes to mRNPs**

5’ repression-decay complexes can be recruited to mRNPs either through their intrinsic RNA affinity or through recruitment by mRNP-specific
factors (see *Poster*). Some decapping factors are recruited by specific RNA sequences or structures in their target mRNAs (Badis et al., 2004; Chowdhury et al., 2007; Dong et al., 2007; Li et al., 2008; Tharun and Parker, 2001). More often, 5’ repression-decay factors interact with mRNA-specific protein complexes that promote translation initiation inhibition, mRNA decay or both. These include the RNA-induced silencing complex (RISC), which silences mRNAs that are targeted by microRNAs (miRNAs) or small interfering RNAs (siRNAs) (Jakymiw et al., 2005; Liu et al., 2005; Pauley et al., 2006), the Upf complex, which targets mRNAs with premature termination codons for nonsense-mediated decay (Durand et al., 2007; Sheth and Parker, 2006), and the related proteins tristetraprolin (TTP) and butyrate response factor 1 (BRF1), which target AU-rich element (ARE)-containing mRNAs for decay (Franks and Lykke-Andersen, 2007). Evidence suggests that RISC-associated GW182 proteins promote RISC-bound mRNP assembly into PBs also independently of 5’ repression-decay factors (Behm-Ansmant et al., 2006; Eulalio et al., 2009).

**Mechanism of PB assembly**

To assemble mRNPs into PBs, PB assembly complexes must release the mRNP from polysomes and promote association with other repressed mRNPs (Anderson and Kedersha, 2009; Balagopal and Parker, 2009; Eulalio et al., 2007a; Franks and Lykke-Andersen, 2008; Kulkarni et al., 2010).
Accordingly, many 5’ repression-decay factors have been implicated in repressing translation initiation (Coller and Parker, 2005; Ferraiuolo et al., 2005; Minshall and Standart, 2004; Tanaka et al., 2006; Tritschler et al., 2010). Given that ribosomes prevent mRNP assembly into PBs, ribosomes probably need to ‘run off’ the repressed mRNA before PB association. In S. cerevisiae, ribosomes can continue translation elongation even after mRNPs have undergone decapping and initiated 5’ to 3’ decay (Hu et al., 2009). Thus, the extent to which individual mRNPs accumulate in PBs is most probably determined not only by their affinity for PB assembly complexes, but also by the relative rates of ribosome run off and mRNA decay. Consistent with this, increased PB formation occurs under conditions that slow mRNA decay rates or enhance translational initiation repression (Coller and Parker, 2005; Cougot et al., 2004a; Fenger-Grøn et al., 2005; Franks and Lykke-Andersen, 2007; Sheth and Parker, 2003).

Evidence indicates that, once ribosomes run off, specific multimerization domains present in PB assembly factors are important for the mRNP to assemble into a PB. Several PB assembly factors contain prion-like glutamine and asparagine (Q/N)-rich regions (see Poster). The best-studied region is in the C terminus of S. cerevisiae Lsm4, a component of the 5’ to 3’ decay-stimulating Lsm1-7 complex. Deletion of this Q/N-rich domain is associated with impaired PB assembly (Decker et al., 2007; Reijns et al., 2008). Although Q/N-rich regions appear to be lacking from most metazoan
Lsm4 proteins, other PB assembly factors contain Q/N-rich regions. Overexpression studies suggest that some of these might play important roles in the assembly of mRNPs into PBs, although further studies are needed to verify this (Behm-Ansmant et al., 2006; Coller and Parker, 2005; Eulalio et al., 2009; Fenger-Grøn et al., 2005; Haas et al., 2010; Jinek et al., 2008; Yu et al., 2005). Other protein domains might also be important, as exemplified by the C-terminal homodimerization domain of the decapping enhancer Edc3, which is important for PB formation in *S. cerevisiae* (Decker et al., 2007; Ling et al., 2008). Important questions for future studies include determining why, as all evidence suggests, PB assembly complexes only assemble into PBs when they are associated with RNA and what prevents polysome-associated mRNPs from assembling into PBs.

**Release of mRNPs from PBs**

The majority of mRNPs in PBs seem to be only transiently associated. FRAP experiments show that many factors rapidly cycle in and out of PBs (Aizer et al., 2008; Andrei et al., 2005; Fujimura et al., 2008b; Kedersha et al., 2005; Leung et al., 2006) and PBs rapidly disappear when new mRNPs are prevented from entering PBs upon treatment of *S. cerevisiae, Drosophila* or human cells with translation elongation inhibitors (Cougot et al., 2004a; Eulalio et al., 2007b; Teixeira et al., 2005). Evidence suggests that the release of mRNPs from PBs involves either mRNA decay or release of the mRNP back
into translation. For example, inhibiting 5’ to 3’ decay can cause enhanced PB accumulation (Cougot et al., 2004a; Fenger-Grøn et al., 2005; Franks and Lykke-Andersen, 2007; Sheth and Parker, 2003). Conversely, PBs are depleted upon reactivation of translation of mRNPs that have been repressed by glucose starvation in *S. cerevisiae* (Brengues et al., 2005) and a specific miRNA-repressed mRNA in human cells has been observed to leave the PBs once repression is lifted (Bhattacharyya et al., 2006). However, some mRNPs might remain static in PBs, as suggested by the slow cycling of some PB factors (Aizer et al., 2008; Andrei et al., 2005; Kedersha et al., 2005; Leung et al., 2006). The factors that determine whether PB-associated mRNPs are targeted for mRNA decay or translational repression alone remain an important subject for future studies.

**PB function**

The functional consequence of the assembly of mRNPs into PBs remains unclear. Manipulations that lead to the loss of visible PBs in *S. cerevisiae*, *Drosophila* or human cells do not disrupt the various mRNA decay pathways tested (Decker et al., 2007; Eulalio et al., 2007b; Stalder and Muhlemann, 2009; Stoecklin et al., 2006). Moreover, no defect in miRNA-mediated translational repression is observed in *Drosophila S2* cells that are depleted of visible PBs (Eulalio et al., 2007b). Although it is difficult to rule out that sub-microscopic PBs exist under such conditions, these observations
suggest that mRNP assembly into macroscopic PBs is not rate limiting for the repression and turnover of these substrates. Future work to determine the protein domains that are crucial to mRNP assembly into PBs and how they are recruited to specific mRNAs might provide important clues to the significance of mRNP assembly into PBs. Currently, it can only be speculated that PBs might serve functions such as sequestering mRNA decay enzymes away from the cytoplasm to prevent promiscuous mRNA decay, concentrating decay enzymes to enhance the kinetics of currently untested mRNA decay pathways or preventing repressed mRNPs from competing for the translation machinery when decay factors are limiting. The conserved ability of eukaryotic cells to assemble mRNPs into PBs suggests that some important function must exist. Delineating this function is a key challenge for future research in the field.

The composition and function of SGs

The composition of mRNPs that assemble into SGs

In contrast to PBs, SGs contain components of the small ribosomal subunit, several translation initiation factors and poly(A)-binding protein. This suggests that mRNPs that assemble into SGs are stalled at a step in translation initiation after the recruitment of a subset of the translation initiation machinery (see Poster). Consistent with this observation, assembly of mRNPs into SGs is induced in a number of conditions that stall translation at the initiation step, including depletion of initiation factors and exposure to cell
stresses or to drugs that impair translation initiation or cause the dissociation of ribosomes (for reviews, see Anderson and Kedersha, 2009; Buchan and Parker, 2009). Of note, knockdown of subunits of eukaryotic translation initiation factor eIF3 inhibits SG formation in human cells, suggesting that this factor plays an important role in SG assembly or that eIF3 depletion stalls the mRNP at a step at which it is not competent for SG assembly (Ohn et al., 2008). It is unclear whether assembly of mRNPs with translation initiation components is a requirement for their assembly into SGs. For example, some translation factors only accumulate in SGs under certain conditions (Buchan et al., 2008; Grousl et al., 2009; Hoyle et al., 2007). Thus, it is likely that mRNPs that assemble into SGs can be stalled at any one of several steps in translation initiation.

**Mechanism of mRNP assembly into SGs**

It is unclear whether all mRNPs that are stalled at a step of translation initiation can assemble into SGs or whether additional factors have to be independently recruited. Factors that can promote mRNP assembly into SGs include the homologous RNA-binding proteins TIA-1 and TIAR (TIA related), and the RasGAP (GTPase-activating protein)-associated endoribonuclease G3BP. All are highly abundant in SGs, and affect SG formation when depleted or overexpressed (Gilks et al., 2004; Kedersha et al., 1999; Ohn et al., 2008; Tourrière et al., 2003). Several other RNA-binding proteins can induce SG
formation when overexpressed, but their role in assembly has not been established (Anderson and Kedersha, 2009; Buchan and Parker, 2009).

Similar to PBs, prion-like domains have been implicated in SG formation. TIA-1 and TIAR both contain a Q/N-rich domain, which, in the case of TIA-1, is required for the spontaneous induction of SGs upon overexpression and can be replaced by the prion-like domain of a heterologous protein (Gilks et al., 2004). The RNA-binding domain of TIA-1 is also required for normal SG formation (Kedersha et al., 1999) suggesting that TIA-1 is directly recruited to mRNPs.

A number of post-translational modifications affect SG assembly. For example, O-linked N-acetylg glucosamine (O-GlcNac)-modified proteins have been found in SGs and depletion of components of the O-GlcNac modification pathway was shown to impair SG assembly (Ohn et al., 2008). In addition, lysine deacetylation (Kwon et al., 2007), arginine methylation (De Leeuw et al., 2007; Dolzhanskaya et al., 2006; Goulet et al., 2008; Hua and Zhou, 2004) and deubiquitylation (Ohn et al., 2008) have all been implicated in SG formation. The relationship between these post-translational modifications and their role in the formation of SG-competent mRNPs remain important points to be addressed in future investigations.

**Possible functions of SGs**
Similar to PBs, the significance of the assembly of mRNPs into SGs remains unclear. Although several factors that are involved in SG formation are translational repressors, there is a lack of clear evidence that the assembly of mRNPs into SGs in itself is important for translational repression (Buchan et al., 2008; Fujimura et al., 2009; Kwon et al., 2007; Loschi et al., 2009; Mokas et al., 2009; Ohn et al., 2008). The stabilization of mRNAs that occurs during many stresses also does not seem to require visible SGs (Buchan et al., 2008). An alternative idea that has been put forth is that mRNPs that are assembled into SGs remain poised to re-enter translation as soon as stress is relieved (Buchan and Parker, 2009). Consistent with this, several studies have presented evidence that the ability to form SGs is correlated with the survival of cells exposed to stress (Buchan and Parker, 2009). Understanding the role of SG formation in mRNP function remains an important goal of future studies.

**Relationship between the mRNPs in PBs and SGs**

Immunofluorescence assays have revealed that PBs and SGs often, but not always, exist in close proximity, a characteristic that can be enhanced when certain SG and PB components are overexpressed (Kedersha et al., 2005). Recent studies in *S. cerevisiae* suggest that PBs can transform into SGs during glucose deprivation, suggesting that, under these conditions, mRNPs in PBs undergo a transition into the mRNPs typically found in SGs (Buchan et al., 2008). This has led to the hypothesis that mRNPs in PBs, SGs
and polysomes represent different steps of a cycle from translationally repressed mRNPs to fully translated mRNPs (see Poster); the formation of PBs and SGs is thus simply a consequence of the accumulation of mRNPs at a specific step of this cycle when a transition becomes rate limiting for a considerable fraction of cellular mRNAs (Balagopal and Parker, 2009; Buchan and Parker, 2009; Franks and Lykke-Andersen, 2008). The transitions in mRNP composition that occur between these steps control whether the mRNA is translated, stored or degraded, and are thus important topics of future investigation.

**Perspectives**

Although several of the factors and conditions that lead to assembly of mRNPs into mRNP granules are now known, the importance of this assembly remains elusive. Because of the highly conserved nature of cytoplasmic mRNP granules in eukaryotes, there is little doubt that the ability to assemble mRNPs into mRNP granules must provide some benefit to cells. Whether this benefit is gained from the ability to form PBs, SGs or more specialized mRNP granules remains to be seen. The rules that govern the formation and disassembly of mRNP granules are likely to be highly similar among granules. Thus, extensive studies of PBs and SGs should help generate tools for investigating the biological importance of assembling mRNPs into various
granules. The role of granule formation in the life of cytoplasmic mRNPs thus remains a key question for future studies.

**Acknowledgements**

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Chapter 2, in full, is a reprint of the material as it appears in Erickson SL and Lykke-Andersen J. Cytoplasmic mRNP granules at a glance. *Journal of Cell Science*, 2011. The dissertation author was the primary author of this paper.
Chapter 3. Hedls enhances decapping enzyme Dcp2 stability and cellular activity by interacting with a novel autoregulatory domain

Summary

mRNA decapping is a central step in eukaryotic mRNA decay, which simultaneously shuts down translation and activates mRNA degradation. A major complex responsible for decapping consists of the decapping enzyme Dcp2 in association with multiple decapping enhancers. An outstanding question is how the activity of Dcp2 is controlled to ensure decapping specifically of mRNAs targeted for degradation, while preventing Dcp2 from acting on non-target mRNAs. Here we identify a C-terminal autoregulatory domain in Dcp2, which prevents Dcp2 activity outside of a decapping complex. The decapping enhancer Hedls (also called Ge-1/Edc4) binds to the Dcp2 autoregulatory domain and thereby promotes Dcp2 stability, decapping complex formation and Dcp2 catalytic activity. This level of regulation may serve to prevent promiscuous decapping and to regulate Dcp2 levels and activity according to cellular needs.

Introduction

Proper control of gene expression requires multiple levels of regulation. In eukaryotic cells, several steps in gene expression are affected by the 5’ N7-methyl guanosine ($^{\text{m7}}$G) cap of mRNAs. The $^{\text{m7}}$G cap is added to RNA
polymerase II transcripts co-transcriptionally, and associates with proteins that in the nucleus stimulate pre-mRNA splicing and RNA nuclear export (Lewis and Izaurralde, 1997). Once an mRNA enters the cytoplasm, the \( \text{m}^7\text{G} \) cap is required for initiation of translation of the majority of mRNAs and at the same time protects mRNAs from degradation from the 5’ end (Cougot et al., 2004b). Thus, the mRNA cap plays an essential role in the processing and function of eukaryotic mRNAs.

Removal of the \( \text{m}^7\text{G} \) cap by the process of decapping is a central step in mRNA turnover, which simultaneously shuts down translation initiation and activates degradation of the mRNA from the 5’ end (Franks and Lykke-Andersen, 2008). The Nudix hydrolase family member Dcp2, is an important cytoplasmic decapping enzyme, which along with its co-activator Dcp1 is conserved amongst eukaryotes (Cohen et al., 2005; Iwasaki et al., 2007; Lykke-Andersen, 2002; van Dijk et al., 2002; Wang et al., 2002; Xu et al., 2006). In addition, another Nudix family enzyme, Nudt16 (also called X29), was recently shown to have cytoplasmic decapping activity in mammals (Li et al., 2011). How the cell controls the activity of decapping enzymes to prevent premature decapping of stable mRNAs, while stimulating decapping of mRNAs destined for degradation is poorly understood.

Several decapping factors have been identified that stimulate the activity of Dcp2 by various mechanisms. For example, the Dcp2 co-factor Dcp1 enhances Dcp2 decapping activity by promoting an activating
conformational change in Dcp2, as evidenced by structural studies of the proteins from yeasts (Deshmukh et al., 2008; Floor et al., 2010; She et al., 2008). Similarly, evidence from *in vitro* studies of yeast decapping factors suggests that multiple decapping enhancers, including Edc3, Pat1, and Scd6, which are all conserved in other eukaryotes, and yeast-specific Edc1 and Edc2, can directly interact with and enhance the catalytic activity of the yeast Dcp2-Dcp1 complex (Borja et al., 2011; Decker et al., 2007; Fromm et al., 2012; Nissan et al., 2010; Schwartz et al., 2003; Steiger et al., 2003). In addition, Pat1 and Scd6, as well as an additional decapping enhancer, the RNA helicase Dhh1, might promote decapping by interfering with the m⁷G cap-associated eukaryotic initiation factor (eIF) 4F complex, as evidenced by the ability of these factors to repress translation initiation (Coller and Parker, 2005; Holmes et al., 2004; Nissan et al., 2010; Pilkington and Parker, 2008). In addition to these decapping enhancers, trans-factors that repress decapping activity have been detected in human cell extracts, including the protein VCX-A (Jiao et al., 2009; Jiao et al., 2006). Despite the current knowledge of these decapping modulators, little is known about how the network of decapping factors control the activity of Dcp2 to act specifically on its mRNA substrates and remain inactive toward non-substrate mRNAs.

A common cellular strategy to prevent uncontrolled activity of enzymes takes advantage of autoregulatory domains that function to prevent enzymes from acting outside of their regulatory complexes. Here we show that the C-
The C-terminus of Dcp2 acts as such an autoregulatory domain, which promotes destabilization of uncomplexed Dcp2 and thereby prevents Dcp2 from activating decapping when unbound by its regulatory factors. The decapping enhancer Hedls (also called Ge-1, Edc4, or Varicose), which facilitates decapping complex assembly in human cells (Fenger-Grøn et al., 2005), binds to the Dcp2 C-terminal autoregulatory domain and, through this interaction, promotes protein stability and catalytic activity of Dcp2. This regulatory mechanism might serve to prevent promiscuous cellular decapping activity and to regulate Dcp2 levels and activity according to cellular needs.

Results

The C-terminus of Dcp2 promotes Dcp2 instability

While performing a deletion study of the Dcp2 protein we observed that deletions in the Dcp2 C-terminus resulted in strikingly enhanced exogenous protein expression as compared to wild-type Dcp2 in human cell lines (data not shown). To test whether this could be a result of the Dcp2 C-terminus promoting instability of the Dcp2 protein, the stability of exogenous Myc-tagged wild-type and deletion mutant Dcp2 proteins were monitored over time after global translation shut-off in human embryonic kidney (HEK) 293T cells. As seen in the Western blots in Figure 3.1A, wild-type Dcp2 is rapidly turned over during a 4-hour time course as compared to HuR, which served as an internal stable protein control (Figure 3.1A, lanes 1-4). By contrast, when the
Figure 3.1: The C-terminus of Dcp2 promotes Dcp2 instability. (A) Western blots of lysates from translation shut-off assays in HEK293T cells transiently expressing wild-type or mutant Dcp2 proteins, as indicated. Time points indicate hours (h) after translational shut-off by cycloheximide (CHX) addition. Endogenous HuR was monitored as a stable protein control. (B) Western blots as in A with addition of protease inhibitor, MG132, when indicated 2 hours (h) prior to translation shut-off with puromycin (Puro). Co-transfected Myc-hnRNP A1 was used as a stable protein control. (C) Graph showing Myc-Dcp2 protein remaining over time normalized to Myc-hnRNP A1.
Dcp2 C-terminal 60 or 120 amino acids are deleted (Dcp2 Δ360-420 and Dcp2 Δ300-420), the resulting truncated Dcp2 proteins are significantly stabilized (Figure 3.1A, lanes 5-8 and 9-12). Quantitative Western blots revealed a half-life of wild-type Dcp2 of 2.9 hours (Figure 3.1B, lanes 1-4; quantified in Figure 3.1C). Dcp2 is significantly stabilized by the addition of MG132, an inhibitor of chymotrypsin-like proteolytic activity, to a half-life that could not be accurately measured because it was longer than that of co-expressed Myc-tagged hnRNP A1, which served as a stable protein control (Figure 3.1B, lanes 5-8, and Figure 3.1C). Thus Dcp2 is targeted for rapid proteolysis in human cell lines dependent on its C-terminus. The Dcp2 C-terminus may only affect protein stability in the context of full-length Dcp2, as we observed no destabilization of DsRed fused with Dcp2 C-terminal amino acids (Figure 3.S1).

**Hedls stabilizes Dcp2 protein**

We had previously observed that steady state levels of exogenously expressed Dcp2 are strongly elevated upon co-expression of Hedls (Fenger-Grøn et al., 2005). We therefore wondered whether Hedls plays a role in Dcp2 stability. A recent report from Bloch et al. mapped the interaction region with Hedls to the C-terminus of Dcp2 (Bloch et al., 2011). Consistent with this, the co-immunoprecipitation assays in Figure 3.2A show complex formation in RNase-treated HEK293T cell extracts of Hedls with wild-type Dcp2, but not
Figure 3.S1: The C-terminus of Dcp2 is not sufficient to target DsRed for degradation. Western blots from lysates of translation shut-off assays using puromycin (Puro) in HEK293Ts cells expressing wild-type and Dcp2 fragments fused to DsRed. Co-transfected Myc-hnRNPA1 served as a stable control.
Figure 3.2: Hedls interacts with the C-terminal region of Dcp2 and stabilizes exogenous Dcp2. (A) Western blots of anti-FLAG immunoprecipitates (IP) from RNase-treated lysates of HEK293T cells co-expressing Myc-tagged wild-type or mutant Dcp2 proteins with FLAG-tagged Hedls. Total input (IN) samples were collected prior to immunoprecipitation. HuR served as a negative control. Mouse light chain IgG was detected in the IP samples as indicated. (B) Western blots of lysates from translation shut-off assays in HEK293T cells expressing Myc-Dcp2 with or without Myc-Hedls. Puromycin (Puro) was added to arrest translation for the indicated number of hours (h). hnRNPA1 was used as a stable control.
with Dcp2 deleted of the C-terminal 60 amino acids (compare lanes 1, 2 with 3, 4). Moreover, indirect immunofluorescence assays revealed that the C-terminal region of Dcp2 is both necessary and sufficient for Dcp2 accumulation in P-bodies in response to Hedls co-expression (Figure 3.S2). Thus, the Dcp2 C-terminus, which promotes Dcp2 instability, is also important for complex formation with Hedls.

To test whether Hedls affects Dcp2 protein stability, we monitored the effect of Hedls co-expression on Dcp2 degradation. As seen in the translation shut-off assay in Figure 3.2B, Dcp2 is markedly stabilized when co-expressed with Hedls, as compared to Dcp2 expressed alone (compare lanes 1-4 with lanes 5-8). Co-expressed hnRNP A1, which was used as a stable protein control, was unaffected by Hedls co-expression (bottom panel). Thus, Hedls specifically stabilizes exogenous Dcp2 protein.

**Hedls complex formation and protein instability functions of the Dcp2 C-terminal domain are separable**

To further investigate the impact of Hedls and the Dcp2 C-terminus on Dcp2 stability, a series of 20-amino acid deletion mutations in the Dcp2 C-terminus were created (Figure 3.3A) and assayed for their effect on the association with co-expressed Hedls in RNase-treated HEK293T cell lysates. As seen in Figure 3.3B, deletion of amino acids 380-400 or 400-420 of Dcp2 prevents co-precipitation with Hedls, while deletion of amino acids 360-380
Figure 3.S2: The C-terminus of Dcp2 is necessary and sufficient for Hedls-mediated localization to PBs. Immunofluorescence of HeLa cells co-expressing Myc-tagged wild-type or mutant Dcp2 with FLAG-Hedls. Anti-Myc antibody was used on the left panels and anti-hDcp1a was used on the right panels as a PB marker. Arrows indicate transfected cells. Percentages of cells with Dcp2 in PBs are listed with the number of cells counted in parentheses.
**Figure 3.3: Conserved hydrophobic residues within the Dcp2 C-terminus are required for Hedls interaction and Dcp2 instability.** (A) Schematic of Dcp2 with the catalytic region in light gray and the C-terminal region in dark gray. Segments that were deleted are delineated with boxes. The conserved Hedls interaction domain is shown with the hydrophobic (Hyd) residues mutated shown in red and shaded. (B) Western blots of immunoprecipitations (IP) from RNase-treated HEK293T cells co-expressing Myc-tagged mutant Dcp2 proteins with FLAG-tagged Hedls. HuR served as a negative control. Input samples (IN) were collected prior to immunoprecipitation. (C) Western blots of lysates from translation shut-off assays using puromycin (Puro) in HEK293Ts cells expressing Dcp2 mutant proteins. Co-transfected Myc-hnRNP A1 was used a stable control.
has no effect (compare lanes 2, 4 and 6). As expected, the negative control protein HuR does not co-purify with Hedls (middle panels).

Orthologs of Hedls are found in metazoans (Eulalio et al., 2007b; Fenger-Grøn et al., 2005; Xu et al., 2006; Yu et al., 2005), whereas no homolog is obvious in yeasts. It is therefore expected that residues important for Hedls interaction should be conserved between metazoan Dcp2 proteins. Comparing the C-terminal region of human Dcp2 with that of Dcp2 from other vertebrates identified five conserved hydrophobic residues within the last 40 amino acids (Figure 3.3A, shown in red and boxed). Similar residues are also found in the C-termini of D. melanogaster and A. thaliana, both of which have characterized Hedls orthologs. To test if these residues are important for Hedls association, all five were mutated to either arginines (Hyd-R) or alanines (Hyd-A). As seen in Figure 3.3B, both of these mutant proteins fail to form a complex with Hedls (compare lanes 8, 10 with 2). This demonstrates the importance of conserved residues in the C-terminal 20 amino acids of Dcp2 for complex formation with Hedls.

To analyze the relationship between Hedls interaction and Dcp2 protein stability, we tested the effect on Dcp2 stability of mutations that prevent Hedls interaction. As seen in the translation shut-off assays in Figure 3.3C (quantified in Figure 3.3D), deletion of the Dcp2 C-terminal 20 amino acids (Δ400-420; lanes 5-8) and mutation of the five conserved residues to alanines (Hyd-A; lanes 9-12), results in significant stabilization of the Dcp2 protein. By
contrast, Dcp2 Δ380-400, which also fails to interact with Hedls (Figure 3.3B), behaves like full-length Dcp2 and is unstable when exogenously expressed (Figure 3.3C, lanes 1-4) but remains unstable when Hedls is co-expressed (Figure 3.S3). These observations taken together suggest that the Dcp2 C-terminal 20 amino acids destabilize the Dcp2 protein, and that assembly of Dcp2 with Hedls, which stabilizes the Dcp2 protein, requires a larger region of the C-terminal domain.

Hedls controls endogenous Dcp2 levels

Experiments described thus far focused on exogenously expressed Dcp2 protein. To assess whether endogenous Dcp2 protein levels are also affected by Hedls, Hedls was depleted using an siRNA and the effect on endogenous Dcp2 levels was monitored. As seen in the Western blots in Figure 3.4A, depletion of Hedls in HeLa cells results in significant reduction of Dcp2 levels compared to control siRNA transfections (compare lane 4 with 5, and with the protein titration in lanes 1-3). Similarly, depletion of Hedls in a different human cell line, HEK 293T, results in reduced Dcp2 levels (Figure 3.4A, compare lane 10 with 6-9). Thus, cellular levels of Dcp2 are dependent on Hedls, and the instability of exogenously expressed Dcp2 likely reflects overexpression of Dcp2 relative to endogenous Hedls levels. Consistent with this, tetracycline-induced expression of exogenous Dcp2 protein causes reduced levels of endogenous Dcp2 in a HEK293 Flp-In T-Rex cell line stably
Figure 3.S3: Hedls interaction is required to stabilize exogenous Dcp2.
Western blots of lysates from translation shut-off assays using puromycin (Puro) in HEK293T cells expressing mutant Myc-tagged Dcp2 constructs with or without Myc-tagged Hedls. Co-transfected Myc-hnRNPA1 served as a stable loading control.
Figure 3.4: Endogenous Dcp2 protein levels are reduced upon Hedls depletion and exogenous Dcp2 expression. (A) Western blots of lysates from HeLa and HEK293T cells transfected with siRNAs targeting luciferase (Luc) as a control, Hedls, or Edc3. Antibodies against endogenous proteins were used to detect Hedls and Dcp2. HuR was used a loading control. Control luciferase samples were titrated at 100%, 33%, and 11% in lanes 1-3 and 6-8. (B) Western blots of lysates from HEK293 Flp-In T cells with tetracycline (TET) inducible Dcp2 with 5 Myc tags stably integrated. 5xMyc-tagged Dcp2 and endogenous Dcp2 were detected using an antibody against endogenous Dcp2. HuR was used as a loading control.
expressing 5xMyc-tagged Dcp2 after a tetracycline-inducible promoter (Figure 3.4B).

**A deadenylated ARE-mRNA decay intermediate accumulates upon Hedls depletion**

Previous studies identified Hedls as a decapping enhancer *in vitro* (Fenger-Grøn et al., 2005; Goeres et al., 2007; Xu et al., 2006). However, cellular overexpression of Hedls in human cells causes defects in decapping (Fenger-Grøn et al., 2005). To determine the role of Hedls on cellular Dcp2 function we tested the effect of Hedls depletion on Dcp2 activity in human cells. A hallmark of a decapping defect is the accumulation of deadenylated intermediates of mRNAs that under normal conditions are targeted for mRNA decay by deadenylation followed by degradation from the 5’ end. As an assay for cellular Dcp2 activity, we therefore used a β-globin reporter mRNA containing the AU-rich element (ARE) from the 3’ untranslated region (3’UTR) of granulocyte-macrophage colony-stimulating factor (GM-CSF) mRNA, because this cis-element is known to recruit enzymes that trigger rapid deadenylation followed by exonucleolytic decay (Chen et al., 1995; Fenger-Grøn et al., 2005; Gao et al., 2001; Lai et al., 2003; Lykke-Andersen and Wagner, 2005). Transcription of the reporter mRNA (β-ARE) was pulsed by removal of tetracycline from human HeLa Tet-off cells transiently transfected with the β-ARE expression plasmid, and the decay of the mRNA was
subsequently followed over time after addition of tetracycline to shut off transcription. An exogenous mRNA (β-GAP) constitutively expressed from a CMV promoter was used as a loading control.

As seen in the Northern blot mRNA decay assays in Figure 3.5A, when Hedls is depleted using an siRNA, a fast migrating β-ARE mRNA band is observed indicative of accumulation of a deadenylated species (lanes 6-8; indicated by β-ARE-A₀). This band is not observed in cells treated with the control Luciferase siRNA (lanes 1-5). Treatment of RNA samples from the first time-points from the Hedls and control knockdown assays with oligo-dT and RNase H confirmed that the faster migrating band observed upon Hedls depletion corresponds to deadenylated mRNA (Figure 3.5B, compare lane 4 with 2). A similarly fast migrating band is observed when decapping is impaired by transient expression of a dominant negative mutant Dcp2 protein (Dcp2 E148Q) (Figure 3.5C, lanes 6-8). The overall decay rate of the β-ARE mRNA was not noticeably affected upon Hedls depletion or expression of Dcp2 E148Q, consistent with degradation from the mRNA 3’ end compensating for the defect in 5’-to-3’ decay (Chen et al., 2001; Mukherjee et al., 2002; Wang and Kiledjian, 2001). These observations demonstrate that depletion of Hedls causes accumulation of a deadenylated ARE-mRNA intermediate similar to that seen when decapping is impaired. This is consistent with the reduction in Dcp2 levels observed upon Hedls depletion.
Figure 3.5: Hedls depletion causes accumulation of deadenylated ARE-mRNA. (A) Northern blots of mRNAs from transcriptional pulse-chase mRNA decay assays in HeLa Tet-off cells expressing a tetracycline repressible β-globin reporter mRNA containing an ARE from GM-CSF mRNA. After a 6-hour pulse, transcription was stopped by addition of tetracycline for the indicated number of minutes (min). β-GAP mRNA was used as a constitutively expressed stable control. Faster migrating deadenylated species are indicated to the right as β-ARE-A₀. (B) Northern blots of samples as in A, treated with or without RNase H and oligo-dT. Deadenylated species are indicated as β-ARE-A₀. (C) Northern blots as in A using cells transfected with a control plasmid or a plasmid expressing catalytically inactive Dcp2 E148Q. A fast migrating band appears when decapping is impaired as indicated by β-ARE-A₀ on the right.
(Figure 3.4A), and suggests that the pool of Dcp2 protein that disappears upon Hedls depletion is an active pool of Dcp2.

**Hedls enhances cellular Dcp2 catalytic activity**

Since Hedls depletion results in decreased Dcp2 levels (Figure 3.4A), the experiments in Figure 3.5 did not reveal whether Hedls plays a role in enhancing Dcp2 activity beyond stabilization of the Dcp2 protein. To directly address this question, we therefore tested the effect on accumulation of the deadenylated β-ARE mRNA of depleting Dcp2 to similar levels as that observed upon Hedls knockdown, using siRNAs directly targeting Dcp2 (Figure 3.6A, compare lane 4 with 5-8). As seen in the Northern blot in Figure 3.6B (quantified in Figure 3.6C), depletion of Dcp2 failed to cause significant accumulation of deadenylated β-ARE mRNA, despite the strong accumulation of this intermediate when Dcp2 is present at similar low levels due to Hedls knockdown (compare lanes 3-6 with 2). The other known cytoplasmic decapping enzyme, Nudt16, is unaffected by Hedls knockdown (Figure 3.5A), demonstrating that the effect of Hedls on decapping is specific to Dcp2. Thus, Hedls stimulates decapping by at least two mechanisms; it prevents the destabilizing effect of the autoinhibitory C-terminal domain of Dcp2, and it stimulates Dcp2 catalytic activity. This could serve as a mechanism to control cellular decapping activity by simultaneously preventing excessive
Figure 3.6: Hedls enhances Dcp2 decapping of ARE-mRNA. (A) Western blots of lysates from HeLa Tet-off cells transfected with siRNAs targeting luciferase (Luc) as a control, Hedls, or Dcp2 and expressing an ARE containing β-globin reporter mRNA and β-GAP stable control mRNA. Antibodies against endogenous proteins were used to probe Hedls, Dcp2, and HuR as a loading control. Control luciferase siRNA treated samples were titrated at 11%, 33%, and 100% in lanes 1-3. A non-specific band detected by the Dcp2 antibody is indicated with an asterisk. (B) Northern blots for β-GAP and β-ARE mRNAs from the samples in A. The fast migrating band appearing in lane 2 is indicated by β-ARE-A₀ on the right. (C) A graphical representation of the signal intensity across each lane in the Northern blot in B, normalized for β-GAP mRNA signal. Hedls siRNA treatment results in an increased signal accumulation for the β-ARE on the right side of the graph indicating detection of the faster migrating band in lane 2 above as compared to lanes 1 and 2-6.
Figure 3.S4: Nudt16 levels remain constant when Hedls is depleted. Western blots of lysates from HeLa Tet-off cells transfected with siRNAs targeting luciferase (Luc) as a control, Hedls or Dcp2. Antibodies against endogenous proteins were used to detect Hedls, Nudt16, and HuR as a loading control. The loading of control luciferase siRNA-treated samples were titrated at 11%, 33%, and 100%.
accumulation of decapping enzyme levels and ensuring that the decapping enzyme is only active when engaged in a decapping complex.

Discussion

Decapping is an important step in mRNA turnover, which simultaneously shuts down translation initiation and initiates exonucleolytic degradation of the mRNA body. Thus, decapping likely needs to be tightly controlled; yet little is known about how decapping enzymes are prevented from engaging mRNAs not targeted for degradation. Here we present evidence that the activity of the human decapping enzyme Dcp2 is controlled by a C-terminal autoregulatory domain, which promotes rapid degradation of uncomplexed Dcp2, while stimulating the activity of Dcp2 when in a decapping complex through association with the decapping enhancer Hedls (Figure 3.7). This could serve as a mechanism to control cellular levels of Dcp2 and prevent promiscuous activation of decapping.

The autoregulatory domain of Dcp2 is located within the C-terminal 20 amino acids, as evidenced by the strong stabilization of Dcp2 that occurs when this region is deleted, or when the conserved hydrophobic amino acids within this region are mutated (Figure 3.3). The mechanism by which the C-terminal domain promotes Dcp2 instability remains unclear. It could either promote a conformation of the Dcp2 protein that renders it vulnerable to proteolysis, or it could serve as a platform for recruitment of cellular protein
Figure 3.7: The C-terminal autoinhibitory domain of Dcp2 is required to degrade free Dcp2 and to enhance the activity of complexed Dcp2. The C-terminus of Dcp2 targets free Dcp2 for degradation until Hedls association occurs. Complexed Dcp2 is not only more stable but also more active demonstrating a key role for the C-terminus in Dcp2 regulation.
degradation machineries. Our observations are more consistent with the former, as the C-terminal 60 amino acids of Dcp2 failed to stimulate rapid degradation when fused with DsRed (Figure 3.S1). Interestingly, the C-terminal region is not important for decapping activity of recombinant Dcp2 in vitro, as the N-terminal 349 amino acids of human Dcp2 were previously found to be sufficient for catalysis, as efficiently and with the same fidelity as full-length Dcp2 (Piccirillo et al., 2003).

The autoinhibitory activity of the Dcp2 C-terminus is counteracted by the decapping enhancer Hedls. The C-terminal 40 amino acids of Dcp2 and conserved residues within are required for Hedls association as evidenced by the lack of Dcp2-Hedls complex formation in the presence of deletions or point mutations within this region (Figure 3.3). The association of Hedls with the C-terminus of Dcp2 enhances cellular Dcp2 activity by two mechanisms (Figure 3.7). First, Hedls stabilizes the Dcp2 protein as observed by the stabilization of exogenous Dcp2 when Hedls is co-expressed (Figure 3.2), and the decrease in endogenous Dcp2 levels upon Hedls depletion (Figure 3.4). Second, Hedls enhances Dcp2 catalytic activity, as shown by the increased accumulation of a deadenylated mRNA decay intermediate when Hedls and Dcp2 are co-depleted using an siRNA targeting Hedls, as compared to conditions in which Dcp2 alone is depleted (Figure 3.6). A stimulatory effect of Hedls on Dcp2 catalytic activity is consistent with previous observations using in vitro assays (Fenger-Grøn et al., 2005; Xu et al., 2006).
How does Hedls promote Dcp2 stability and activity? By binding to the C-terminal region of Dcp2, Hedls could competitively inhibit the recruitment of protein degradation machineries and at the same time promote the recruitment of additional decapping-stimulating factors to the Dcp2 complex. Alternatively, Hedls could promote a conformational change in Dcp2, which renders it catalytically active and stable. Our previous observation that Hedls promotes the assembly of Dcp2 with other decapping factors, including Dcp1 (Fenger-Grøn et al., 2005), is consistent with both of these scenarios. Either way, our studies reveal a mechanism by which uncomplexed cellular Dcp2 is kept unstable and inactive. This could provide a mechanism for tightly controlling cellular decapping activity in order to prevent promiscuous decapping and resulting uncontrolled repression of cellular mRNAs. Future studies should reveal whether this type of regulation explains the observation that Dcp2 is differentially expressed in mouse tissues and throughout development (Song et al., 2010). In addition, it remains to be determined whether the other mammalian decapping enzyme Nudt16 is under similar tight control.

Methods

Plasmid Constructs

Expression plasmids, created using derivatives of pcDNA3 (Invitrogen), for tetracycline-regulated expression of β-globin ARE-mRNA decay (β-ARE) reporter, and constitutively expressed β-GAP internal control mRNA, as well
as for N-terminally Myc- and Flag-tagged Dcp2, Dcp2 E148Q, Hedls, DsRed and hnRNP A1 have been previously described (Fenger-Grøn et al., 2005; Lykke-Andersen, 2002; Lykke-Andersen et al., 2000; Lykke-Andersen and Wagner, 2005; Singh et al., 2007). Plasmids expressing Myc-Dcp2 containing deletion or point mutations were created using the QuickChange Site-Directed mutagenesis method (Stratagene). Myc-DsRed-Dcp2 fusions were created by subcloning DsRed into the BamHI site N-terminal to the Dcp2 start site in the described Dcp2 vectors; sequences available upon request.

**Antibodies**

Rabbit polyclonal antisera against hDcp1a (Lykke-Andersen and Wagner, 2005), and Hedls (Fenger-Grøn et al., 2005), and mouse monoclonal anti-HuR 3A2 (Gallouzi et al., 2000) have been previously described. Rabbit anti-Dcp2 and rat anti-Nudt16 were generous gifts from Dr. Megerditch Kiledjian (Song et al., 2010; Wang et al., 2002). Mouse monoclonal anti-Myc-tag 9B11 and anti-Flag M2 were purchased from Cell Signaling and Sigma, respectively. For Western blotting, antibodies were used at the following dilutions in TBST (100 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05% Tween 20) containing 5% milk: α-hDcp1a 1:2,000; α-Hedls 1:1,000; α-HuR 1:25,000; α-Dcp2 1:400; α-Nudt16 1:200; α-Myc 1:1,000; and α-Flag 1:1,000.

**siRNAs**
All siRNAs were purchased from Dharmacon. Luciferase (Luc) control siRNA: 5’-CGUACGCGGAAUACUUCGAU-3’ + 5’-
UCGAAGUAUUCGCUACGU-3’; Hedls ORF siRNA: 5’-
GAGUUAAAGAUGUGUGUAUU-3’ + 5’-UACACCACAUCUUUAACUCUU-3’;
Hedls 3’UTR siRNA 1, ON-TARGET modified: 5’-
CACUGAAGGCCAGCAGACAU-3’ + 5’-UGUCUGCUAGGCCUUCAGUGU-3’;
Hedls 3’UTR siRNA 2: 5’- GUGUGGUAGUCAGAAGGUUU-3’ + 5’-
AACCUCUGACUACCACUU-3’; hEdc3 siRNA: 5’-
GCACUGAAAUAAGUCGAAU-3’ + 5’-UUCAGCUUUUAUUCAGUGC-3’;
Dcp2 ORF siRNA pool: siGENOME SMARTpool-M008425; Dcp2 3’UTR
siRNA pool: ON-TARGETplus SMARTpool-L008425.

mRNA Reporter Assays and Northern blots

Pulse-chase mRNA decay assays were performed as described earlier (Lykke-Andersen and Wagner, 2005). HeLa Tet-off cells (Clontech) cultured in 3.5-cm wells in 2 ml of Dulbecco Modified Eagle Medium (DMEM, Invitrogen) supplemented with 10% Fetal Bovine Serum (FBS, Invitrogen) and 1% Penicillin-Streptomycin solution (PS, Invitrogen) were transfected 72 hours before reporter mRNA induction with siRNAs at a final concentration of 20 nM using siLentFect Lipid Reagent (Bio-Rad) according to the manufacturer’s protocol. In Dcp2 siRNA titration experiments in Figure 3.6A, the total amount of siRNA was adjusted to 20 nM with addition of Luc siRNA. The next day,
cells were transfected with plasmids using TransIT-HeLaMONSTER transfection reagent (Mirus) per manufacturer’s protocol and maintained in 50 ng/ml of tetracycline to repress transcription of reporter mRNAs. Cells in **Figure 3.5C** were transfected with 1 µg β-ARE, 50 ng β-GAP, and, for samples in lanes 6-10, with 2.5 µg Myc-Dcp2 E148Q expression plasmids, with empty pcDNA3 vector to a total of 4 µg. Cells in **Figures 3.5A, 3.6A, and 3.6B** were transfected with 1 µg β-ARE, and 30 ng (or 50 ng for **Figure 3.5A**) β-GAP expression plasmids in addition to empty pcDNA3 vector to a total of 2 µg. Two days later (three days in **Figure 3.5C**), to induce β-ARE mRNA transcription, cells were washed once with Phosphate-Buffered Saline (PBS; 137 mM NaCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, 2.7 mM KCl, pH 7.4) and incubated in DMEM/10% FBS/1% PS lacking tetracycline for 6 hours, before tetracycline was added back at 1 µg/ml to stop transcription and start the chase experiment. The first time point (t = 0) was collected 30 minutes after tetracycline addition to ensure complete transcriptional arrest. Total cellular RNA was prepared by addition of TRIZOL Reagent (Invitrogen) directly to cells followed by RNA isolation as per manufacturer’s protocol, and analyzed by Northern blotting as previously described (Lykke-Andersen and Wagner, 2005). Deadenylated control RNA samples were produced using oligo-dT₂₄ and RNase H (NEB) as described previously (Clement et al., 2011). Total cellular protein was collected after washing cells once with PBS by addition of Sodium Dodecyl Sulfate (SDS) load buffer (100 mM Tris-HCl pH 6.8, 200 mM
DTT, 4% SDS, 0.2% bromophenol blue, and 20% glycerol) directly to cells, followed by rigorous pipetting to shear genomic DNA. Protein samples were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) followed by Western blotting, to assess exogenous protein expression and siRNA knockdown efficiencies.

**Translation shut-off Assays**

Human Embryonic Kidney (HEK) 293T cells seeded in 2.2-cm wells in 1 ml of DMEM/10% FBS/1% PS were transfected using TransIT-293 transfection reagent (Mirus) per manufacturer’s protocol. Cells in Figure 3.1A were transfected with 0.8 µg wild-type or mutant Myc-Dcp2 expression plasmids and 0.2 µg empty pcDNA3 vector. Cells in Figures 3.1B and 3.4A were transfected with 1.25 µg wild-type or mutant Myc-Dcp2 and 50 ng Myc-hnRNP A1 expression plasmids, and empty pcDNA3 vector to a total of 1.5 µg. Cells in Figure 3.2A were transfected with 50 ng Myc-hnRNP A1 expression plasmid, and 1.45 µg Myc-Dcp2 (lanes 1-4), or 1.25 µg Myc-Dcp2 plus 0.2 µg Myc-Hedls expression plasmids (lanes 5-8). Two days after transfection, translation was arrested with puromycin (Sigma) at 5 µg/ml or cycloheximide (Sigma) at 50 µg/ml, and total cellular protein was isolated at times indicated in figures by addition of SDS load buffer as described above. In Figure 3.1B, the protease inhibitor MG132 (Sigma) was added at 10 µM for 2 hours prior to translational arrest. In Figure 3.2C, steady state protein levels were analyzed
after siRNA transfection, as described above, with a second transfection 48 hours after the first and samples collected 48 hours later. Samples were analyzed by SDS-PAGE followed by Western blotting. Quantification of signal intensities for half-life calculations was performed using ECL Plus Detection Reagent (Amersham) and scanned on a Typhoon Trio (Amersham).

Co-immunoprecipitation Assays

Immunoprecipitation assays in Figures 3.2A and 3.3C were performed as described previously (Lykke-Andersen, 2002). HEK293T cells seeded in 10-cm plates in 10 ml DMEM/10% FBS/1% PS were co-transfected using TransIT-293 transfection reagent (Mirus) with 2 µg Flag-Hedls expression vector and either 7 µg Myc-Dcp2 Δ360-420 plus 6 µg empty pcDNA3 vector, or 13 µg of other Myc-Dcp2 expression vectors as indicated in figures. 48 hours after transfection, cells were washed with 10 ml PBS and then lysed by incubating cells on ice for 10 minutes in 800 µl of ice-cold hypotonic lysis buffer (0.1% Triton X-100, 10 mM Tris-HCl pH 7.5, 10 mM NaCl, 2 mM EDTA, 1 µM aprotinin (Sigma), 1 µM leupeptin (Sigma), and 1 mM phenylmethylsulfonyl fluoride (Sigma)). Lysates were incubated on ice for another 5 minutes with RNase A (Sigma) added to 125 µg/ml and NaCl added to 150 mM. Cell debris was removed by centrifugation at 14,000 rpm for 15 minutes at 4°C, and an input sample was collected before the remaining supernatant was nutated for 2-3 hours at 4°C with 40 µl of anti-FLAG M2
agarose (Sigma). Beads were washed 8 times with NET-2 buffer (50 mM Tris-HCl pH 6.5, 150 mM NaCl, 0.05% Triton-X100). Protein complexes were eluted with 30 µl SDS load buffer and analyzed by SDS-PAGE followed by Western blotting.

**Indirect Immunofluorescence Assays (in supplemental material)**

Indirect immunofluorescence assays were performed as previously described (Fenger-Grøn et al., 2005). HeLa cells grown in 2.2-cm wells in 1 ml DMEM/10% FBS/1%PS were transfected using TransIT-HeLaMONSTER transfection reagent (Mirus) with 0.8 µg Myc-Dcp2 expression constructs and 0.2 µg Flag-Hedls or empty pcDNA3 vector. After 24 hours, cells were trypsinized and seeded in 8-well Lab-Tek chamber slides (Thermo Scientific/Nunc). Two days after transfection, cells were fixed by incubation at room temperature for 10 minutes with 4% paraformaldehyde in PBS and washed twice in PBS before permeabilizing and blocking cells by incubation for 15 minutes in a solution containing PBS, 1% goat serum (Sigma), and 0.5% Triton X-100. Cells were washed 3 times with PBS/1% goat serum and incubated with the following antibody dilutions in PBS/1% goat serum for 1 hour or more: α-hDcp1a 1:200; α-Myc-tag 9B11 1:100; and α-Flag M2 1:100. After washing twice, secondary antibodies, Alexa Fluor 488 donkey anti-mouse IgG and Texas Red-X goat anti-rabbit IgG (Molecular Probes), were added at 1:1,000 for 40 minutes. Cells were washed 4 times with PBS/1%
goat serum and once with water before slides were coated with Vectashield Mounting Medium (Vector Labs) and sealed with a coverslip.

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Chapter 3, in full, is currently being prepared for submission for publication of the material as (has been submitted for publication of the material as it may appear in) Erickson SL, Corpuz EO, Maloy J, Fillman C, and Lykke-Andersen J. Hedls enhances decapping enzyme Dcp2 stability and cellular activity by interacting with a novel autoregulatory domain. The dissertation author is the primary investigator and author of this paper.
Chapter 4. Is Dcp2 stability controlled by mRNA substrate levels?

Introduction

Decapping mediated by Dcp2 is a critical step in several mRNA decay pathways. In order to decap, Dcp2 must make contacts with the RNA and the 5’ cap (Piccirillo et al., 2003; van Dijk et al., 2002). Dcp2 binds RNA nonspecifically through weak ionic interactions with an RNA binding channel (Deshmukh et al., 2008), but can also be recruited to specific mRNAs. RNA binding proteins involved in silencing mediated by AU-rich elements (AREs), miRNAs, and premature termination codons recruit mRNA decay machinery including Dcp2 to activate mRNA decay (Franks and Lykke-Andersen, 2008). In addition, mRNAs have been identified that contain a 5’ step loop in close proximity to the cap that directly recruits Dcp2 with high affinity (Li et al., 2008). Therefore, the control of Dcp2 activity may be tightly linked to its recruitment to the proper mRNA substrates.

The decapping complex protein Hedls is required for the accumulation of Dcp2 in RNA-dependent cytoplasmic foci called processing bodies (PBs) (Figure 3.S2). These granules contain mRNPs targeted for silencing by the 5’ decay machinery including the specific pathways mentioned above (see Chapter 2). I identified an autoregulatory domain in Dcp2, which promotes increased protein stability and cellular activity when associated with Hedls (Chapter 3), but under which conditions Hedls stabilizes Dcp2 and promotes
its activity is still unknown. One intriguing possibility is that Dcp2 is stabilized when it is associated with substrate mRNPs, which could serve as a mechanism to control cellular Dcp2 levels according to the abundance of substrate mRNAs. The results from my studies addressing this model are presented below.

**Results**

**Dcp2 is concentrated in PBs by the C-terminus of Hedls.**

To study whether Dcp2 is stabilized by mRNPs, I utilized several strategies. PBs were used as a cellular localization assay to observe when Dcp2 is concentrated with mRNPs targeted for 5’ decay. Hedls fragments were created by cloning the N-terminal (Nt, 1-724) and C-terminal (Ct, 725-1401) halves of the Hedls open reading frame into expression vectors (Figure 4.1A). Hedls localization was monitored by indirect immunofluorescence assays of HeLa cells transfected with the FLAG-tagged Hedls constructs. hDcp1a was used as an endogenous PB marker. Arrows indicate transfected cells and percentage of cells with FLAG-Hedls co-localization with hDcp1a are indicated with the number of cells counted in parentheses. As observed previously (Fenger-Grøn et al., 2005), overexpressed Hedls concentrates in enlarged PBs (Figure 4.1B, top panels), suggesting that Hedls overexpression causes accumulation of translationally repressed mRNPs. In contrast, the N-terminus of Hedls fails to concentrate in PBs and displays a diffuse cytoplasmic staining
Figure 4.1: The C-terminus of Hedls promotes Dcp2 concentration in PBs. (A) Schematic of Hedls fragments cloned into expression vectors. N-terminal amino acids 1-714 (Nt). C-terminal amino acids 715-1401 (Ct). (B) Indirect immunofluorescence of HeLa cells expressing FLAG-tagged wild-type or Hedls fragments. Anti-FLAG antibody was used on the left panels and anti-hDcp1a was used on the right panels as a PB marker. Arrows indicate transfected cells. Percentages of cells with Hedls in PBs are listed with the number of cells counted in parentheses. (C) Indirect immunofluorescence as in B, with cells co-expressing FLAG-tagged Hedls constructs with Myc-tagged Dcp2. Anti-Myc antibody was used on the left panels and anti-hDcp1a was used on the right panels.
A

WD40 repeat region

Hedls

Nt

Ct

1

724

1401

B

α-FLAG-Hedls

100% (4)

α-hDcp1a

α-FLAG-Hedls

0% (20)

α-FLAG-Hedls Nt

40% (35)

α-FLAG-Hedls Ct

α-Myc-Dcp2

100% (100)

α-hDcp1a

0% (100)

α-FLAG-Hedls Nt

62% (117)

α-FLAG-Hedls Ct

Myc-Dcp2
(Figure 4.1B, middle panels). Interestingly, hDcp1a localization also seems to be similarly affected in cells transfected with the N-terminus of Hedls. This region could facilitate hDcp1a concentration in PBs although the regions of Hedls that interact with hDcp1a are unknown. The C-terminus of Hedls was found to concentrate in PBs in 40% of cells (Figure 4.1B, bottom panels). These results are consistent with results published while these studies were in progress (Yu et al., 2005).

Since Hedls concentrates Dcp2 in PBs (Figure 4.1C, top panel and Figure 3.S2), I next addressed how the Hedls fragments affect Dcp2 localization. HeLa cells were co-transfected with FLAG-tagged Hedls constructs and Myc-tagged Dcp2 expression constructs. Indirect immunofluorescence assays were performed to visualize Dcp2 localization using hDcp1a as a PB marker. I observed that, as expected, the N-terminus of Hedls is unable to concentrate Dcp2 in PBs (Figure 4.1C, middle panels), while the C-terminus of Hedls concentrates Dcp2 in PBs in 62% of the cells (Figure 4.1C, bottom panels). These observations suggest that full-length Hedls and, to a lesser extent, the C-terminus of Hedls support association of Dcp2 with repressed mRNPs that accumulate in PBs.

Both the N-terminus and the C-terminus of Hedls bind Dcp2

In the previous study, I found that co-expression of Hedls with Dcp2 stabilizes Dcp2 only if they interact with one another (Figure 3.S4). To assay
whether Dcp2 stability changes when it is co-expressed with Hedls fragments, which differentially localize to PBs, I first addressed whether these fragments can bind Dcp2. RNase-treated lysates from human embryonic kidney (HEK) 293T cells co-transfected with Myc-tagged Hedls constructs and FLAG-tagged Dcp2 constructs were subjected to anti-FLAG immunoprecipitations to assay for co-purifying Myc-tagged proteins. As seen in the Western blots in Figure 4.2A, both Hedls fragments co-purify with FLAG-Dcp2 (lanes 2 and 4). Myc-tagged hnRNPA1 served as a negative control and did not co-purify as expected.

Hedls was found to self-associate via its C-terminus in A. thaliana while our studies were underway (Xu et al., 2006). To determine regions of Hedls self-association in human cells, HEK293T cells were co-transfected with Myc- and Flag-tagged Hedls constructs and subjected to co-immunoprecipitation assays as above. Similar to observations in A. thaliana, I show in Figure 4.2B that full-length Hedls co-purifies with itself and the C-terminal Hedls fragment (lanes 2 and 6). In addition, the C-terminal Hedls fragment alone strongly co-purifies with itself (Figure 4.2B, lane 8). In contrast, the N-terminal Hedls fragment does not co-purify with full-length Hedls above background (Figure 4.2B, lane 4). Myc-hnRNPA1 and Myc-Upf1 served as negative controls and did not co-purify with Hedls.

**N-terminal and C-terminal Hedls fragments stabilize Dcp2**
**Figure 4.2: Hedls N-terminal and C-terminal fragments bind Dcp2.** (A) Western blots of total inputs (IN) and anti-FLAG immunoprecipitates (IP) from RNase-treated lysates of HEK293T cells co-expressing FLAG-tagged Dcp2 and Myc-tagged Hedls fragments. hnRNPA1 served as a negative control. Mouse heavy chain IgG was detected where indicated. (B) Western blots of total inputs and anti-FLAG immunoprecipitations (IP) as described above from cells co-expressing Myc-tagged and FLAG-tagged Hedls constructs.
I determined that the N-terminus of Hedls cannot concentrate in PBs or self-associate, but still interacts with Dcp2. To test the effect that this fragment has on Dcp2 stability, I monitored protein degradation over time after global translation shut-off assays in HEK293T cells co-transfected with Myc-tagged Dcp2 and Myc-tagged Hedls constructs. In three independent experiments, one of which is shown in Figure 4.3, Dcp2 appears to be stable when co-expressed with any of the Hedls fragments (compare Dcp2 levels with that of the internal hnRNPA1 control). In contrast, Dcp2 expressed alone is unstable as previously observed (Figure 4.3, right panel). Therefore, both N- and C-terminal Hedls fragments appear to bind and stabilize Dcp2 regardless of their ability to accumulate Dcp2 in PBs.

**Catalytically inactive Dcp2 accumulates in PBs similarly to wild-type**

Next I tried an alternative way to test if Dcp2 is stabilized by association with mRNPs using a catalytically inactive point mutant (Dcp2-E148Q) (Lykke-Andersen, 2002) that may result in the recruitment of Dcp2 to mRNPs, but a failure to release due to a lack of activity. This was based on preliminary data from the lab showing that the recycling of GFP-hDcp1a in and out of PBs, as measured by fluorescence recovery after photobleaching (FRAP) assays, is significantly decreased when Dcp2-E148Q is co-transfected (J. Dennis, personal communication). Dcp2 was later found to be relatively static in PBs as it did not recover after photobleaching for the duration of the experiment.
Figure 4.3: Dcp2 is stabilized by the N-terminus and C-terminus of Hedls. Western blots of lysates from translation shut-off assays using puromycin. HEK293T cells co-expressing Myc-tagged Dcp2 and Hedls constructs were collected at the indicated hours (hrs) after translation shut-off. Co-transfected Myc-hnRNPA1 was used as a stable control.
(Aizer et al., 2008). Because of this, I decided to instead analyze the accumulation of wild-type versus inactive Dcp2 in PBs to assess whether inactive Dcp2 is trapped with repressed mRNPs targeted for decay.

Indirect immunofluorescence was performed with HeLa cells transfected with Myc-Dcp2 constructs using hDcp1a as an endogenous PB marker. Arrows indicate transfected cells. I found that in cells expressing low levels of Myc-Dcp2, Dcp2 co-localizes in foci with hDcp1a, but cells with higher expression levels show a diffuse Dcp2 cytoplasmic staining and a reduction in hDcp1a foci (Figure 4.4, top panels). This is consistent with previous results from our lab (Fenger-Grøn et al., 2005). A similar pattern was observed with Myc-Dcp2-E148Q indicating that this mutant is not trapped with PB mRNPs when overexpressed (Figure 4.4, bottom panels). Unlike for wild-type Dcp2, there appears to be no decrease in hDcp1a PBs in high expressing Dcp2-E148Q cells though, which supports the FRAP data that hDcp1a cycles less quickly from PBs when inactive Dcp2 is co-expressed. Thus, the majority of the overexpressed mutant Dcp2 pool is not trapped, but the fraction of protein that is bound with mRNPs (and hDcp1a) might be trapped.

**Catalytically inactive Dcp2 is unstable**

Since PBs are dynamic accumulations of decay intermediates that depend on the rate of decay compared to the abundance of targets (see Chapter 2), Dcp2 may be still stabilized by mRNPs that are not observed in
Figure 4.4: Catalytic inactivation of Dcp2 does not alter its cellular distribution in PBs. Indirect immunofluorescence of HeLa cells expressing Myc-Dcp2 (top panels) or Myc-Dcp2-E148Q (bottom panels). Anti-Myc antibody was used to detect Dcp2 on the left panels and hDcp1a was used as a PB marker in the middle panels. Arrows indicated cells expressing low or higher levels of Myc-Dcp2 constructs. The white box is a zoomed image of the dotted white box.
PBs. To test whether inactive Dcp2 is more stable than wild-type Dcp2, translation shut-off assays were performed in HEK293T cells. The Western blot in Figure 4.5 shows the previously observed rapid degradation of Myc-Dcp2 (see also Chapter 3) compared to the stable Myc-hnRNPA1 control. Similarly Myc-Dcp2-E148Q is also turned over quickly during the time course (Figure 4.5, right panels). Therefore, catalytic inactivation of Dcp2 does not increase its stability. However, the mechanisms of Dcp2 recruitment to mRNAs and release after decapping are currently not well understood so it cannot be ruled out that this mutant protein is not trapped on mRNPs. It is also possible that the exogenous protein is in excess over its mRNP substrates.

**Rrp41 stem loop containing mRNA reporters do not stabilize Dcp2**

Recently, a stem loop structure was identified at the 5’ end of Rrp41 mRNA that directly recruits Dcp2 with high affinity (Li et al., 2008). Characterization of this stem loop showed that the structure, not the sequence, is important and must be within 10 nucleotides from the cap to bind and promote decapping (Li et al., 2009). In order to more directly test whether mRNA itself can stabilize Dcp2, I inserted this 60 nucleotide stem loop near the 5’ end of both tetracycline inducible (TET) and constitutively expressed (CMV) β-globin mRNA reporters. Since the transcription start site for the used vectors could change when different sequences are inserted, insertions were made at different locations (Figure 4.6A).
Figure 4.5: Catalytically inactive Dcp2 is unstable. Western blots of lysates from translation shut-off assays in HEK293T cells transiently expressing Myc-tagged wild-type or mutant Dcp2 proteins, as indicated. Time points indicate hours (hrs) after translational shut-off by puromycin addition. Co-transfected Myc-hnRNPA1 was used as a stable protein control. Experiment performed by my honors student, Jeff Maloy.
Figure 4.6: Dcp2 remains unstable when co-expressed with Rrp41 stem loop containing mRNA substrates. (A) DNA sequence for the 60 nucleotide Rrp41 stem loop inserted into the β-globin constructs. The underlined residues indicate the location of insertion into the vector with the predicted transcription start site in bold lettering for each construct. (B) Western blots of lysates from translation shut-off assays using puromycin in HEK293T cells co-transfected with Myc-Dcp2 and β-globin reporter mRNAs with or without the Rrp41 stem loop inserted at various positions near the 5' transcription start site. Myc-hnRNPA1 was used as a stable control. The asterisk indicates a background band observed in some experiments.
A

Rrp41 stem loop insertion:
ACCTCCGGAAACCGTAGATTCCGGGCGGCCTCAGGAGCCCGGGGACGCTAGTCTCTCCGGCG

Insertion sites:
TATATAAGCGAGCTCCTCTGCTTAACCTGCGGGACCTCCGGAAACCGT - Rrp41-49
TATATAAGCGAGCTCCTCTGCTTAACCTGCGGGACCTCCGGAAACCGT - Rrp41-45
TATATAAGCGAGCTCCTCTGCTTAACCTGCGGGACCTCCGGAAACCGT - Rrp41-42
TATATAAGCGAGCTCCTCTGCTTAACCTGCGGGACCTCCGGAAACCGT - Rrp41-39

B

<table>
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<th>Rrp41-45</th>
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<td>0</td>
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<td>hrs</td>
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background
Myc-Dcp2

Myc-hnRNPA1

<table>
<thead>
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<th>CMV β-reporter:</th>
<th>wt</th>
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<th>Rrp41-45</th>
</tr>
</thead>
<tbody>
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<td>0</td>
<td>2</td>
<td>4</td>
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<td>ional arrest</td>
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background
Myc-Dcp2

Myc-hnRNPA1
To test the effect of increasing Dcp2 substrate levels on Dcp2 protein stability, translation shut-off assays were performed on HEK293T cells co-transfected with plasmids expressing the indicated mRNA substrate, Myc-Dcp2, and Myc-hnRNPA1 as a stable control. I found that Myc-Dcp2 is rapidly turned over in cells expressing any of the substrate mRNAs similar to when no reporter is transfected (Figure 4.6B). Northern blot analysis showed that all reporters expressed in cells (data not shown and Figure 4.7A). Therefore, Dcp2 is not stabilized by expression of these reporter mRNAs either because they were not recruiting a large fraction of the Dcp2 protein pool, or because this mRNA cannot stabilize Dcp2.

To test whether these mRNAs are actual substrates for Dcp2-mediated mRNA decay in my assays, I performed pulse chase mRNA decay assays in HeLa Tet-off cells transfected with the tetracycline-inducible β-globin or Rrp41 stem loop reporter constructs and siRNAs to knock down either control luciferase (Luc) or Dcp2. After pulsing reporter mRNA transcription by removing tetracycline, total mRNA samples were collected over time after transcriptional shut-off by tetracycline addition and analyzed by Northern blotting to follow their rates of decay. Constitutively expressed β-GAP mRNA was used as a stable control. In Figure 4.7A, I observed that the half-lives ($t_{1/2}$) of most of the reporters are increased with Dcp2 knockdown, although the control knockdown half-lives are all longer than the time course making it difficult to know how accurate these numbers are. Surprisingly, I did not
**Figure 4.7: Rrp41 reporter mRNA half-lives are similar to the wild-type reporter.** (A) Northern blots of mRNAs from transcriptional pulse-chase mRNA decay assays in HeLa Tet-off cells expressing tetracycline repressible β-globin reporter mRNAs with or without the Rrp41 stem loop inserted at various positions. After a 9 hour pulse, transcription was stopped by addition of tetracycline for the indicated number of minutes (min). β-GAP mRNA was used as a constitutively expressed stable control. Half-lives (t1/2) were calculated using the Typhoon and Image Quant Software. (B) Western blots of cell lysates from A using endogenous Dcp2 and HuR antibodies to confirm siRNA depletion of Dcp2.
observe a shorter half-life for any of the constructs with the Rrp41 stem loop insertions as compared to the βwtβ control lacking the insertions (Figure 4.7A). siRNA depletion of Dcp2 was confirmed by western blotting using endogenous HuR as a control (Figure 4.7B).

The published half-life of endogenous Rrp41 in HEK293T cells is 3.5 hours and 7 hours with Dcp2 knockdown, while the half-life of an electroporated in vitro transcribed RNA containing the stem loop is 1 hour with the stem loop and 2.2 hours without (Li et al., 2008). My reporters are showing significantly longer half-lives which could be explained by the difference in the expression system or the stem loops may not be positioned within 10 nucleotides from the cap. Future studies will need to be performed to determine whether Dcp2 is binding to my substrate and whether the overall concentration of Dcp2 targets is higher after expression of the substrates before conclusions can be made about whether Dcp2 stability is influenced by cellular mRNA substrate concentrations.

Discussion

Decapping prevents the recruitment of the translational machinery and is critical for several mRNA decay pathways that regulate gene expression. This underscores the importance of regulating decapping activity in cells, but little is currently understood about this regulation. In the previous study (Chapter 3), I identified a C-terminal autoregulatory domain in Dcp2, which
affects its stability and activity through interactions with Hedls. In an attempt to
dissect the mechanism of Dcp2 stabilization, I tested how mRNA substrate
levels affect Dcp2 stability. This is an intriguing model for Dcp2 stabilization
because Dcp2 function is linked to its recruitment to mRNAs. In addition, Hedls
stabilizes Dcp2 and causes Dcp2 concentration with mRNPs in PBs. Using
Hedls mutants that fail to concentrate Dcp2 in PBs, I observed that Dcp2
stability does not correlate with its concentration in PBs (**Figures 4.1 and 4.3**).
Catalytic inactivation of Dcp2 also does not affect its stability (**Figure 4.5**).
Finally, I found that co-transfection of a reporter mRNA that has been
previously shown to recruit Dcp2 with high affinity does not affect its stability
(**Figure 4.6B**). Thus, I have no evidence so far that Dcp2 stability is regulated
according to mRNA substrate levels. However, more experiments are needed
to rule out this possibility. Therefore, the mechanism of Dcp2 stabilization is
still not known, but could be very important for maintaining appropriate
decapping activity in the cells and preventing degradation of non-substrate
mRNAs.

One way to test if RNA binding stabilizes Dcp2 is through altering
cellular Dcp2 substrate levels. I attempted to do this with the Rrp41 stem loop
reporters (**Figure 4.6A**), but as mentioned above these unexpectedly do not
decrease the half-lives of the mRNA reporter constructs (**Figure 4.7A**). It will
be important to use a sequencing method such as 5’ RACE to determine
whether the stem loop is positioned within 10 nucleotides from the 5’ cap. This
distance affects not only decapping of the RNA, but also the actual binding of Dcp2 (Li et al., 2009), which would be important if Dcp2 is stabilized by the mRNA substrate. Even more importantly, Dcp2 RNA co-immunoprecipitations should be performed with these substrates to determine whether Dcp2 has an increased affinity compared to other mRNAs. This method for determining whether mRNA substrates stabilize Dcp2 may prove difficult because it depends on finding the correct level of mRNA substrate compared to Dcp2. The ratio of Dcp2 to mRNA substrates is not known and is likely dynamic due to the nature of the enzyme’s activity in mRNA decay.

Another, perhaps more direct way to test this model, is to alter the mRNA binding capacity of Dcp2 itself. In yeast, structural studies have mapped an RNA binding channel comprised of positively charges residues important for RNA binding (Deshmukh et al., 2008). These are not well conserved at the sequence level, but modeling revealed a similar channel with positively charged residues in human Dcp2. In cells, a modest growth phenotype was found after mutating several of these residues. Thus, creating this mutant may be challenging, but could provide evidence for whether or not mRNA binding plays a role in Dcp2 stability.

Finally, Hedls may be stabilizing Dcp2 independent of mRNA association. A discussion of how this might occur is found in the previous section (Chapter 3). Future studies are required to tease out the mechanism of Dcp2 stabilization and will lend insight into when and how Dcp2 is regulated.
Methods

Plasmid Constructs

Derivatives of pcDNA3 (Invitrogen), were used to create expression plasmids for tetracycline-regulated expression of β-globin mRNA decay reporters, and constitutively expressed β-GAP internal control mRNA, as well as for N-terminally Myc- and Flag-tagged Dcp2, Dcp2 E148Q, Hedls, and hnRNP A1 that have been previously described (Fenger-Grøn et al., 2005; Lykke-Andersen, 2002; Lykke-Andersen et al., 2000; Lykke-Andersen and Wagner, 2005). Plasmids for tetracycline-regulated expression of β-globin mRNA decay reporters with the Rrp41 stem loop insertion (see primers below) and plasmids expressing Myc-Dcp2 containing deletions were created using the QuickChange Site-Directed mutagenesis method (Stratagene). Hedls fragments were subcloned into pcDNA3 constructs using the corresponding cDNA sequences.

Primers

All primers were purchased from Invitrogen. Rrp41-5’ UTR-F(forward):

5’-ACCTCCGGAAACCGTAGATTCCGGGCGGTCGGAGCCGCCGGGAGCTGTagTTCTCCCGCTAGCTACTATTGCT-3’; Rrp41-R(reverse)49: 5’-ACCGTTTCCCGAGGTTCCGAGTTCAGCCAGAGCTCTGTGTTTATA-3’;

Rrp41-R45: 5’- ACGGTTTCCCGAGGTTCCGAGTTCAGCCAGAGCTCTGTGTTT
ATATA-3'; Rrp41-R42: 5'-ACGGTTTCCGAGGTTAGTTAGCCAGAGAGCTCT
GCTTATATA-3'; Rrp41-39: 5'-ACGGTTTCCGAGGTTAGCCAGAGAGCTCT
GCTTATATA-3'.

**Antibodies**

Rabbit polyclonal antisera against hDcp1a (Lykke-Andersen and Wagner, 2005) and mouse monoclonal anti-HuR 3A2 (Gallouzi et al., 2000) have been previously described. Rabbit anti-Dcp2 was a generous gift from Dr. Megerditch Kiledjian (Wang et al., 2002). Mouse monoclonal anti-Myc-tag 9B11 and anti-Flag M2 were purchased from Cell Signaling and Sigma, respectively. For Western blotting, antibodies were used at the following dilutions in TBST (100 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05% Tween 20) containing 5% milk: α-HuR 1:25,000; α-Dcp2 1:400; α-Myc 1:1,000; and α-Flag 1:1,000.

**siRNAs**

All siRNAs were purchased from Dharmaco. Luciferase (Luc) control siRNA: 5'-CGUACGCAGAUACUCGAU-3' + 5'
UCGAAGUAUUCGCAGCUU-3'; Dcp2 ORF siRNA pool: siGENOME SMARTpool-M008425.

**Indirect Immunofluorescence Assays**
Indirect immunofluorescence assays were performed as previously described (Lykke-Andersen and Wagner, 2005). HeLa cells grown in 2.2-cm wells in 1 ml Dulbecco Modified Eagle Medium (DMEM, Invitrogen) supplemented with 10% Fetal Bovine Serum (FBS, Invitrogen) and 1% Penicillin-Streptomycin solution (PS, Invitrogen) were transfected using TransIT-HeLaMONSTER transfection reagent (Mirus). Cells in Figure 4.1B and C were transfected with 0.2 µg Flag-Hedls expression constructs and 0.8 µg Myc-Dcp2 or empty pcDNA3-Myc vector. Cells in Figure 4.4 were transfected 0.7 µg Myc-Dcp2 constructs, 0.3 µg empty pcDNA5 FLAG vector, and 0.25 µg pSuperPuro and were incubated with 2mg/ml puromycin (Sigma) to select transfected cells. After 24 hours, cells were trypsinized and seeded in 8-well Lab-Tek chamber slides (Thermo Scientific/Nunc) Two days after transfection, cells were fixed by incubation at room temperature for 10 minutes with 4% paraformaldehyde in Phosphate-Buffered Saline (PBS; 137 mM NaCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, 2.7 mM KCl, pH 7.4) and washed twice in PBS before permeabilizing and blocking cells by incubation for 15 minutes in a solution containing PBS, 1% goat serum (Sigma), and 0.5% Triton X-100. Cells were washed 3 times with PBS/1% goat serum and incubated with the following antibody dilutions in PBS/1% goat serum for 1 hour or more: α- hDcp1a 1:200; α-Myc-tag 9B11 1:100; and α-Flag M2 1:100. After washing twice, secondary antibodies, Alexa Fluor 488 donkey anti-mouse IgG and Texas Red-X goat anti-rabbit IgG (Molecular Probes), were added at 1:1,000
for 40 minutes. Cells were washed 4 times with PBS/1% goat serum and once with water before slides were coated with Vectashield Mounting Medium (Vector Labs) and sealed with a coverslip.

**Co-immunoprecipitation Assays**

Immunoprecipitation assays were performed as described previously (Lykke-Andersen, 2002). Human Emybryonic Kidney (HEK) 293T cells seeded in 3.5-cm plates in 2 ml DMEM/10% FBS/1% PS were co-transfected using TransIT-293 transfection reagent (Mirus) with 0.4 µg Myc-Hedls expression vectors and 1.6 µg FLAG-Dcp2 plus 0.2 µg Myc-hnRNPA1 and 0.2 µg Myc-Upf1 vectors in Figure 4.2A. Cells in Figure 4.2B were transfected with 0.6 µg of both FLAG- and Myc-Hedls constructs with 0.3 µg Myc-hnRNPA1 and 0.3 µg Myc-Upf1 plasmids. 48 hours after transfection, cells were washed with 1 ml PBS and then lysed by incubating cells on ice for 10 minutes in 400 µl of ice-cold hypotonic lysis buffer (0.1% Triton X-100, 10 mM Tris-HCl pH 7.5, 10 mM NaCl, 2 mM EDTA, 1 µM aprotinin (Sigma), 1 µM leupeptin (Sigma), and 1 mM phenylmethylsulfonyl fluoride (Sigma)). Lysates were incubated on ice for another 5 minutes with RNase A (Sigma) added to 125 µg/ml and NaCl added to 150 mM. Cell debris was removed by centrifugation at 14,000 rpm for 15 minutes at 4°C, and an input sample was collected before the remaining supernatant was nutated for 2 hours at 4°C with 40 µl of anti-FLAG M2 agarose (Sigma). Beads were washed 8 times with NET-2 buffer (50 mM Tris-
HCl pH 6.5, 150 mM NaCl, 0.05% Triton-X100). Protein complexes were eluted with 20 µl NET-2 buffer with 1 mg/ml FLAG Peptide (sigma) for 4 hours at 4˚C. Sodium Dodecyl Sulfate (SDS) load buffer (100 mM Tris-HCl pH 6.8, 200 mM DTT, 4% SDS, 0.2% bromophenol blue, and 20% glycerol) was added to 20 µl of the collected eluate and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) followed by Western blotting.

**Translation shut-off Assays**

HEK293T cells seeded in 2.2-cm wells in 1 ml of DMEM/10% FBS/1% PS were transfected using **TransIT-293** transfection reagent (Mirus) per manufacturer’s protocol. Cells in **Figure 4.3** were transfected with 0.8 µg Myc-Dcp2, 0.2 µg Myc-Hedls expression plasmids or 0.2 µg empty vector, and 25 ng Myc-hnRNPA1. Cells in **Figure 4.5** were transfected with 1.5 µg wild-type or mutant Myc-Dcp2 and 0.1 µg Myc-hnRNP A1 constructs. Cells in **Figure 4.6B** were transfected with 1 µg Myc-Dcp2 and 0.5 µg TET-mRNA reporter plasmids with 0.2 µg Myc hnRNPA1 or 0.4 µg CMV-mRNA reporters with 0.1 µg Myc-hnRNPA1. Cells in **Figure 5.1** were seeded in 1.1 cm wells in 500 ml of media and were transfected with 25 ng Myc-hnRNPA1 and 0.5 µg Myc-Dcp2, 0.5 µg Myc-3K-R, or 0.1 µg Myc-1-360 and 0.425 µg pcDNA3-Myc. Two days after transfection, translation was arrested with puromycin (Sigma) at 5 µg/ml and total cellular protein was isolated at the indicated times by addition of SDS load buffer directly to cells, followed by rigorous pipetting to shear
genomic DNA. Samples were analyzed by SDS-PAGE followed by Western blotting.

**mRNA Reporter Assays and Northern blots**

Pulse-chase mRNA decay assays were performed as described earlier (Lykke-Andersen and Wagner, 2005). HeLa Tet-off cells (Clontech) cultured in 3.5-cm wells in 2 ml of DMEM/10% FBS/1%PS were transfected 48 and 96 hours before reporter mRNA induction with siRNAs at a final concentration of 20 nM using siLentFect Lipid Reagent (Bio-Rad) according to the manufacturer’s protocol. At 48 hours, cells were also transfected with plasmids using TransIT-HeLaMONSTER transfection reagent (Mirus) per manufacturer’s protocol and maintained in 50 ng/ml of tetracycline to repress transcription of reporter mRNAs. Cells in **Figure 4.7** were transfected with 1 µg β-globin reporter plasmids, and 60 ng β-GAP expression plasmids in addition to pcDNA3-Myc vector to a total of 4 µg. Two days later, to induce β-globin reporter mRNA transcription, cells were washed once with PBS and incubated in DMEM/10% FBS/1% PS lacking tetracycline for 9 hours, before tetracycline was added back at 1 µg/ml to stop transcription and start the chase experiment. The first time point (t = 0) was collected 30 minutes after tetracycline addition to ensure complete transcriptional arrest. Total cellular RNA was prepared by addition of TRIZOL Reagent (Invitrogen) directly to cells followed by RNA isolation as per manufacturer’s protocol, and analyzed by
Northern blotting as previously described (Lykke-Andersen and Wagner, 2005). Total cellular protein was collected after washing cells once with PBS by addition of SDS load buffer as described above. Protein samples were analyzed by SDS-PAGE followed by Western blotting, to assess siRNA knockdown efficiency in Figure 4.7B.

Acknowledgements

I would like to thank my honors student, Jeff Maloy, for providing the western data in Figure 4.5.
Chapter 5. Conclusions and Future Directions

Conclusions

mRNA decapping prevents translation initiation and activates mRNA decay, which are salient regulators of gene expression. A major unresolved question is how decapping of target substrates is promoted and activation of decapping of other mRNAs is prevented. In these studies, I identified an autoregulatory domain in the C-terminus of Dcp2, which regulates its stability and activity through interactions with the decapping enhancer Hedls (Chapter 3). This may provide an important means to modulate decapping activity in cells. In addition, I tested whether Dcp2 is stabilized by mRNA substrates as a mechanism to maintain proper decapping levels in the cell (Chapter 4). Although these studies showed no evidence of this mechanism of Dcp2 regulation, my results provide a stepping stone for futures studies investigating how decapping is regulated, which are discussed below.

Future Directions

How does the C-terminus of Dcp2 trigger degradation?

I observed that the C-terminus of Dcp2 is required for its instability (Chapter 3), but it is currently unclear what is responsible for this degradation. One candidate is the proteasome. The drug MG132, which inhibits chymotrypsin-like proteolytic activity including the activity of the proteasome,
was shown to stabilize Dcp2 in Chapter 3 (**Figure 3.1B**). Proteins targeted for
degradation by the proteasome are often polyubiquitinated at lysine residues
(Ciechanover, 2005). To test whether lysine residues in the C-terminal 60
amino acids of Dcp2 are required for degradation, I made conservative
mutations of all three lysines to arginines (3K-R). Protein degradation was
followed over time after global translation shut-off assays in HEK293T cells
expressing Myc-tagged wild-type or mutant Dcp2 proteins. As seen in the
Western blots in **Figure 5.1**, the lysine mutant degrades similarly to wild-type
Dcp2 in contrast to the stable C-terminal deletion mutant. Co-transfected Myc-
hnRNPA1 served as a stable control. Therefore, the lysines in the C-terminus
are not required for degradation. Future studies are required to determine
whether alternate lysines can be polyubiquitinated. For example, ubiquitin
blots could be performed on wild-type Dcp2 immunoprecipitated from cells
treated with MG132 to block degradation or co-immunoprecipitation assays
could be performed with Dcp2 and tagged ubiquitin.

There are also other mechanisms for targeting proteins for degradation
by the proteasome. Inherently unstructured regions have been found to
undergo ubiquitin-independent degradation by the 20S core of the proteasome
(Asher et al., 2006). Using bioinformatics, Gunawardana et al. identified a
highly unstructured region in the C-terminus of Dcp2 in *A. thaliana*
(Gunawardana et al., 2008). The unstructured nature of the C-terminus may
be important for targeting the protein for degradation, although this remains to
Figure 5.1: Mutation of the C-terminal lysines in Dcp2 does not affect its stability. Western blots of lysates from translation shut-off assays using puromycin. HEK293T cells expressing Myc-tagged Dcp2 constructs were collected at the indicated hours (hrs) after translation shut-off. Co-transfected Myc-hnRNPA1 was used as a stable control. The three lysines in the C-terminus of Dcp2 were mutated to arginines (3K-R).
be tested in plants or human cells. Structural studies on the human Dcp2 have not been performed and would be particularly useful to see if Dcp2 has different conformations and whether Hedls influences Dcp2 structural changes. The above studies will also aid in determining whether Dcp2 is targeted for degradation by the proteasome or other proteases in the cell.

How does Hedls enhance the activity of Dcp2?

I presented evidence that Hedls stimulates Dcp2 catalytic activity. There are many possible mechanisms by which Hedls could be enhancing the activity of stabilized Dcp2 protein. These include promoting decapping complex assembly, recruitment to mRNA targets, catalysis of decapping, or any combination of the above. The simplest hypothesis, which could encompass all of the processes above, is that the autoregulatory domain of Dcp2 actively inhibits the protein by promoting an inactive conformation until Hedls association causes a shift to an active conformation. It has been suggested that yeast Dcp2 activity is regulated by conformational changes facilitated by Dcp1 (Floor et al., 2010; She et al., 2008), and it is possible that Hedls plays a similar role in metazoans. The inactive conformation of Dcp2 could titrate away decapping factors or compete for binding to targets as perhaps indicated by the dominant negative effect seen with the inactive point mutant of Dcp2 (Figure 3.6C). Therefore, subsequent targeting of the inactive
conformation for degradation could be important to ensure that Dcp2 outside of the decapping complex is removed from the cell.

Alternatively, Hedls could play distinct roles in activating Dcp2 by stabilizing the protein and then enhancing the steps above by a different mechanism. A role for Hedls in promoting decapping complex formation is supported by a previous study from our lab, which revealed that exogenous Dcp2 only co-purifies with hDcp1a when Hedls is co-expressed and co-purifies with Edc3, an additional decapping enhancer, only when both Hedls and hDcp1a are co-expressed (Fenger-Grøn et al., 2005). A recent study from Fromm et al. demonstrated that enhancers, Edc3 and Scd6, interact with yeast Dcp2, but this domain is not conserved in metazoans and may be instead present in Dcp1 (Fromm et al., 2012). This suggests that the C-terminus of Dcp2 in metazoans might be critical for recruiting decapping complex proteins by recruiting Hedls followed by Dcp1 and its associated factors. The only other decay factor known to co-purify with Dcp2 in human cells is Pat1, which also interacts with Hedls and many factors involved in translational silencing, deadenylation, and decapping (Chapter 1). Therefore, Dcp2 interaction with Hedls and Pat1 may be critical for its association with other decay factors. Alternatively, Hedls may be indirectly required to cause a conformational change in Dcp2, which then allows assembly of the decapping complex.

In order to decap, Dcp2 must be recruited to the mRNA. Dcp2 has a general RNA binding domain in the N-terminus, but it is not clear how
important this domain is for recruitment to specific substrates or whether additional proteins are needed in cells. Li et al. identified a subset of mRNAs that contain a stem-loop that recruits Dcp2 with high affinity (Li et al., 2009; Li et al., 2008). In addition, several highly regulated mRNAs have motifs that recruit RNA binding trans-factors that associate with the decay machinery (Franks and Lykke-Andersen, 2008). Although Dcp2 associates with several trans-factors, it is not clear exactly how Dcp2 is recruited. Hedls could facilitate such recruitment, but its interactions with mRNAs or trans-factors are currently unknown.

Finally, RNA helicases have been recently found to play a role in mRNA decay by remodeling the mRNP, which is required for further degradation (Franks et al., 2010). Therefore, Hedls may recruit helicases such as the decapping activator Rck/p54 to release decay factors from the mRNP to allow access of the 5'-phosphatase by the 5'-exonuclease Xrn1. Several proteins that antagonize decapping also associate with the mRNA cap in human cells (Jiao et al., 2009; Jiao et al., 2006; Khanna and Kiledjian, 2004). Hedls could play a role in facilitating the released of these proteins to allow Dcp2 access. Future studies that tease out the mechanistic details of the regulation of the decapping process including which steps are affected by either Hedls or the C-terminus of Dcp2 and whether regulatory conformational changes occur in Dcp2 in metazoans will be important areas of investigation.
Are other decay enzyme complexes regulated similarly to the Dcp2 decapping complex?

mRNA decay often requires the contribution of several mRNA decay enzymes, many of which exist in multi-subunit complexes. The human exosome, which is involved in both mRNA processing and 3’ to 5’ mRNA decay, is comprised of 9 distinct proteins that form a capped ring structure and 2 additional catalytic subunits, hDIS3L and hRRP6. Complex variants have been identified in different subcellular compartments, which could be regulated by the relative expression levels of various factors (Lykke-Andersen et al., 2011). It would be interesting to determine whether the catalytically active components of exosome complexes are regulated by protein turnover similar to Dcp2.

Deadenylases complexes also exist in eukaryotic cells. The Ccr4-Not complex is comprised of 9 proteins including an E3 ubiquitin ligase and two deadenylases, Ccr4 and Pop2, which each have an additional human ortholog. Pan2 deadenylase exists in a complex with Pan3. It is thought that deadenylation may occur by coordinating the activities of both of these complexes. Other deadenylases exist that are differentially expressed and/or have substrate specificity, but are not known to be in a complex (Garneau et al., 2007). The extent to which complex variants form and how that regulates their activity is not well understood. In addition, how the E3 ubiquitin ligase in the Ccr4-Not complex affects deadenylation is not known, but could provide a
means to target specific factors in the complex for degradation to regulate
deadenylation.

An additional decay enzyme that may be regulated by complex
formation is Nudt16, which was recently found to have cytoplasmic decapping
activity in mammals (Song et al., 2010). Currently, no associated proteins
have been identified and interactions with known decapping factors have not
been tested. Nudt16 and Dcp2 contain homologous Nudix motifs and have
overlapping and distinct substrates (Li et al., 2011) suggesting that they could
form similar and distinct complexes to accomplish this substrate specificity. To
bioinformatically determine whether the C-terminal autoregulatory domain of
Dcp2 is conserved in Nudt16, I performed a sequence alignment as seen in
Figure 5.2. There appears to be conservation of the distribution of 4 of the 5
hydrophobic residues that I found to be important for Dcp2 stability and
interaction with Hedls (Figure 5.2, residues indicated in red). Translation shut-
off assays and Hedls co-immunoprecipitation assays with wild-type Nudt16
and Nudt16 with mutations in this domain will aid in determining if Nudt16 is
regulated in a similar manner to Dcp2. In addition, if Nudt16 binds to Hedls
through these conserved residues, Nudt16 and Dcp2 might compete for
binding, influencing which enzyme is active.

Further characterization of these complexes including how their
catalytic subunits are regulated will be critical to understanding how mRNA
decay proceeds. Perhaps the stability and/or activity of these enzymes is
Figure 5.2: Alignment of the Dcp2 autoregulatory domain with a similar region in Nudt16. The schematics of Dcp2 and Nudt16 indicate the catalytic Nudix domains in light gray and region of conservation with the Dcp2 autoregulatory domain in dark gray. The residues important for Dcp2 stability and Hedls interaction are shown in red (top sequence). The analogous hydrophobic residues in Nudt16 are also shown in red (bottom sequence).
differentially regulated based on which factors they associate with, similar to how Dcp2 is affected by Hedls. This could be an exciting way to regulate specific targets under various conditions.

**Is Dcp2 expression regulated according to varying cellular conditions?**

Gene expression is highly regulated so that cells can differentiate and develop into an organism that can respond to changing environments. mRNA decay plays an important role in this regulation. For example, when the ARE element that recruits mRNA decay factors including Dcp2 is deleted from the 3’ UTR of the mRNA encoding the cytokine tumor necrosis factor-alpha (TNFα), this induces overexpression of the protein and development of a systemic inflammatory syndrome (Kontoyiannis et al., 1999). Thus, it is possible that the modulation of Dcp2 activity is important to differentially regulate gene expression to respond to changing environments.

Recently, a Dcp2 hypomorphic mouse was created to determine how Dcp2-mediated decapping contributes to overall mRNA decay in an organism. Dcp2 down-regulation alone was not sufficient to induce the systemic inflammation described above or any other obvious phenotypes (Song et al., 2010). This may be due to the residual Dcp2 expression observed, redundant decapping enzymes, and/or a compensatory increase in decay from the 3’ end. In support of the redundancy of mRNA decay mechanisms present in mammals, developmental defects have been found in organisms where Dcp2
is the only identified decapping enzyme. In *C. elegans*, increases in embryonic lethality were found when both Dcp1 and Dcp2 were depleted using RNAi (Lall et al., 2005) and a mutant disrupted in the Dcp1 gene of *D. melanogaster* has the embryo abdominal deletion phenotype (Lin et al., 2006). In addition, deletions in Dcp1, Dcp2, or Hedls in *A. thaliana* cause post-embryonic lethality (Xu et al., 2006). Finally, deletion of Dcp2 in *S. cerevisiae* causes a severe slow growth phenotype (Dunckley and Parker, 1999) and Dcp1 deletions in *S. pombe* are inviable (Sakuno et al., 2004). In sum, cellular growth and development in many organisms are affected by loss of Dcp2.

While Dcp2 expression may not be essential for a mammalian organism, it could be important to prevent overexpression of Dcp2. My data that Dcp2 is an unstable protein and can be regulated by interactions with the decapping complex suggest that regulation of the expression of Dcp2 might be important. Interestingly, Dcp2 was found to display tissue specific expression in adult mouse and human tissues, with the highest expression in the brain and testes but no expression in the liver. In addition, Dcp2 protein expression decreases dramatically in the heart, liver, and kidney over the course of development from an embryo to an adult mouse (Song et al., 2010). How this tissue specific down-regulation of Dcp2 protein is achieved is not known. One possibility is that the autoregulatory domain of Dcp2 is required to decrease mRNA decapping levels in specific tissues of the adult. One mechanism for this could be by regulation of Hedls expression, but it has not been tested
whether Hedls is differentially regulated during development or in different tissues. Future studies will lend tremendous insight into how Dcp2 is regulated and how this contributes to biological processes in organisms.
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