# *Caenorhabditis elegans* Decapping Proteins: Localization and Functional Analysis of Dcp1, Dcp2, and DcpS during Embryogenesis

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Though posttranscriptional regulation is important for early embryogenesis, little is understood regarding control of mRNA decay during development. Previous work defined two major pathways by which normal transcripts are degraded in eukaryotes. However it is not known which pathways are key in mRNA decay during early patterning or whether developmental transcripts are turned over via specific pathways. Here we show that *Caenorhabditis elegans* Dcp2 is localized to distinct foci during embryogenesis, reminiscent of P-bodies, the sites of mRNA degradation in yeast and mammals. However the decapping enzyme of the 3' to 5' transcript decay system (DcpS) localizes throughout the cytoplasm, suggesting this degradation pathway is not highly organized. In addition we find that Dcp2 is localized to P-granules, showing that Dcp2 is stored and/or active in these structures. However RNAi of these decapping enzymes has no obvious effect on embryogenesis. In contrast we find that nuclear cap binding proteins (CBP-20 and 80), eIF4G, and PAB-1 are absolutely required for development. Together our data provides further evidence that pathways of general mRNA metabolism can be remarkably organized during development, with two different decapping enzymes localized in distinct cytoplasmic domains.

# INTRODUCTION

Although pathways of normal mRNA degradation are being increasingly defined in multiple species (reviewed in Coller and Parker, 2004), little is known about the contribution of these pathways to development. Indeed although it would seem that timely mRNA turnover might be critical to the sculpting of specific gene expression patterns during embryogenesis, it is not known whether transcript degradation during development occurs along the general mRNA turnover pathways or is a more specific process. Furthermore, although recent data from both yeast and human cells suggests that mRNA degradation pathways are more highly organized at the cell biological level than had previously been realized (Ingelfinger et al., 2002; van Dijk et al., 2002; Sheth and Parker, 2003; Cougot et al., 2004b), the subcellular organization of these processes in intact metazoa or during embryogenesis remains uncharacterized.

Degradation of normal transcripts is thought to occur along two major pathways, conserved in multiple eukaryotes. The first step in mRNA decay is common to both pathways and involves shortening of the poly(A) tail (Decker and Parker, 1993; Lumelsky, 1995; Zuk and Jacobson, 1998; Wilusz *et al.*, 2001). Although both mRNA turnover systems involve subsequent exonuclease-mediated

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degradation of the body of the transcript, as well as metabolism of the mRNA cap structure (m<sup>7</sup>GpppN) by decapping enzymes, these processes are carried out by distinct sets of enzymes in different temporal orders.

In the 5' to 3' decay pathway, deadenylation is followed by Dcp1/Dcp2 mediated decapping, which exposes the body of the transcript to Xrn-1, a 5' to 3' exonuclease (Decker and Parker, 1993; Hsu and Stevens, 1993; Muhlrad et al., 1994; Beelman et al., 1996; Dunckley and Parker, 1999). This pathway was first described in yeast and was subsequently found to be active in human cells and nematodes (Couttet et al., 1997; Wang et al., 2002b; Cohen et al., 2004). Although it appears that specific transcripts are degraded along this pathway, it is known that the 3' to 5' decay system can mediate mRNA turnover of these transcripts when 5' to 3' decay is inhibited (see for example, Muhlrad et al., 1995; Beelman et al., 1996). This may explain why deletions of the dcp1 or dcp2 gene are viable in yeast (Beelman et al., 1996; Dunckley and Parker, 1999). Indeed, although decreasing function of Xrn-1 by RNA interference (RNAi) in Caenorhabditis elegans is embryonic lethal, such lethality is not fully penetrant, and dying *C. elegans* embryos get as far as ventral epithelial closure upon depletion of Xrn-1 (Newbury and Woollard, 2004).

The 3' to 5' decay pathway begins with degradation of the deadenylated mRNA body by a complex of proteins known as the exosome (Anderson and Parker, 1998; van Hoof and Parker, 1999; Wang and Kiledjian, 2001; Butler, 2002; Mukherjee *et al.*, 2002). The resulting mRNA cap structure is then turned over by a distinct decapping enzyme known as DcpS (Wang and Kiledjian, 2001; Liu *et al.*, 2002). This seems

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to be the dominant transcript decay pathway in vitro in mammalian cells as well as in a nematode system and also appears to play a substantial role in mRNA degradation in yeast (Chen *et al.*, 2001; Wang and Kiledjian, 2001; Mukherjee *et al.*, 2002; He *et al.*, 2003; Cohen *et al.*, 2004).

Recently, cell biological data has emerged indicating that components of the 5' to 3' decay pathway show punctate cytoplasmic localization. In particular Saccharomyces cerevisiae Dcp2 is found to localize to specific cytoplasmic sites, called processing or P-bodies (Sheth and Parker, 2003). Two to three P-bodies are observed in yeast cytoplasm, and these have been shown to be sites to which mRNAs undergoing decay are targeted. It has also been shown that Dcp2 as well as Xrn-1 are found in multiple cytoplasmic foci in mammalian cell lines (Bashkirov et al., 1997; Ingelfinger et al., 2002; Lykke-Andersen, 2002; van Dijk et al., 2002; Cougot et al., 2004a; Liu et al., 2004; Andrei et al., 2005; Kedersha et al., 2005). In addition recent data suggests that micro-RNA targets and components of the RNA induced silencing complex localize to P-bodies (Ding et al., 2005; Liu et al., 2005; Sen and Blau, 2005). Thus it appears that components of the 5' to 3' decay pathway show an unexpectedly high level of subcellular organization. This organization and concentration of mRNA metabolism proteins in cytoplasmic foci has been compared with P-granules and stress granules (Wickens and Goldstrohm, 2003). The former are particles observed during early development. Although the precise function of P-granules is not clear, they are observed in C. elegans germ cells and are partitioned at each of several early divisions into the germ-line precursor (Strome and Wood, 1982, 1983). Studies have shown that P-granules are enriched in a variety of mRNAs (Seydoux and Fire, 1994; Pitt et al., 2000; Schisa et al., 2001). In addition to transcripts, many proteins potentially involved in mRNA translation and modification are found in P-granules. For example, the vasa-like RNA helicases GLH-1-4 are present in P-granules and are involved in germ-line development (Gruidl et al., 1996; Kuznicki et al., 2000). In addition poly(A) polymerase has also been localized to P-granules (Wang et al., 2002a), as have translation initiation factors (Amiri et al., 2001; Hanazawa et al., 2004) as well as Sm proteins (Barbee et al., 2002). In addition Pgranules contain CGH-1, a helicase that is homologous to an enhancer of decapping in yeast, Dhh-1 (Navarro et al., 2001). Thus P-granules are sites containing mRNAs as well as proteins involved in message regulation and have been analogized to cytoplasmic P-bodies (Wickens and Goldstrohm, 2003; Coller and Parker, 2004).

The processes of mRNA decapping and decay are complicated during C. elegans development, because nematodes have populations of mRNAs with distinct cap structures, an important issue when considering the function of proteins that interact with the methylated 5' cap structure in this model system. More specifically 70% of C. elegans transcripts have a 22-nucleotide sequence (called the spliced leader or SL) trans-spliced to their 5' end (Zorio et al., 1994). The spliced leader sequence brings with it a trimethylated cap structure (m<sup>2,2,7</sup>GpppG, TMĞ), as opposed to the monomethylated m<sup>7</sup>GpppN cap (MMG) found on un-transspliced mRNAs (Thomas et al., 1988; Liou and Blumenthal, 1990; Van Doren and Hirsh, 1990). The presence of two populations of mRNAs with distinct 5' structures has profound implications for proteins that recognize the mRNA cap. Indeed it has previously been shown that C. elegans has five isoforms of the cap-binding translation initiation factor, each with distinct cap-binding eIF4E. properties (Jankowska-Anyszka et al., 1998; Keiper et al., 2000). IFE-1, 2, and 5 behave redundantly during development, but it is not clear whether other cap interacting proteins are also functionally dispensable during embryogenesis (Keiper *et al.*, 2000).

To extend our previous analysis of nematode Dcp2 function to the cell biological level and to determine whether the subcompartmental localization present in yeast and human cells is also evident in intact metazoa, we have examined the localization of Dcp2 during development. We find that Dcp2 localizes to foci in the somatic and germ-line precursor cells (P-cells). Colocalization with GLH-1 and other markers suggests that in addition to these small cytoplasmic foci, Dcp2 is found in P-granules. This localization is compromised in *pgl-1* mutants (*bn101, bn102,* and *ct131*). In contrast we find that the decapping enzyme of the 3' to 5' mRNA decay pathway, DcpS, is found throughout the cytoplasm, indicating that this mRNA decay pathway is apparently not organized at foci. Although it is thought that metabolism of the mRNA cap is essential because it might poison translation of mRNA or be misincorporated into nucleic acids, we find that the decapping enzymes are not essential for development. Knocking down dcp1, dcpS, or xrn-1 function does not obviously affect Dcp2 cytoplasmic foci. However, RNAi targeting ccf-1 (ortholog of a S. cerevisiae deadenylase complex component) leads to alterations in Dcp2 immunostaining. Unlike the decapping enzymes and most of the nematode eIF4E genes, we find that other proteins involved in mRNA cap interaction (nuclear cap-binding complex, eIF4G, and PAB-1) are absolutely essential for development. Thus, having previously examined mRNA degradation during nematode embryogenesis in a biochemical manner (Cohen et al., 2004), we have extended this analysis to examine the cell biological organization and functional requirement for mRNA decapping during development.

# MATERIALS AND METHODS

#### Nematode Strains

C. elegans N2 were used as the wild-type strain. In addition *pgl-1* mutant strains were obtained from the *C. elegans* stock center (http://biosci.uum.edu/CGC/CGChomepage.html) and were SS2, SS579, and SS580 (Kawasaki *et al.*, 1998), *glh-1* mutant strains used were VC178 and RB656 and were also obtained from the CGC stock center (*Caenorhabditis* Genetics Center, http://biosci.uum.edu/CGC). *C. elegans* were kept at 25°C unless otherwise stated. Temperature sensitive *pgl-1* and *glh-1* mutant strains were kept at 16°C (permissive temperature) and then moved to 25°C to reveal the mutant phenotype.

#### Protein and Antibody Production

Recombinant C. elegans Dcp2 and DcpS proteins were previously described (Cohen et al., 2004, 2005). Proteins were produced by inoculating 1 L of LB medium with Rosetta (DE3) cells (Novagen, Madison, WI) transformed with the above plasmids. The culture was grown at 37°C until it reached  $O.D_{600}$  0.6. Protein production was induced by adding 0.4 mM isopropyl β-D-thiogalactopyranoside (IPTG) and by shaking the bacterial culture for either 4 h at 37°C or overnight at 25°C. Proteins were purified as previously described, by passing over a Ni2+-nitrilotriacetic acid-agarose column (Qiagen, Valencia, CA) or using glutathione-Sepharose 4B beads (Amersham, Piscataway, NJ). To obtain pure Dcp2 protein, the column purified Dcp2 protein was separated from smaller copurifying products by electrophoresis on a 5% Criterion Tris-HCl gel (Bio-Rad, Hercules, CA) and stained briefly in Coomassie blue. After briefly destaining, the 76-kDa band was excised from the gel in as small a volume as possible and used for inoculation. Polyclonal antibodies were raised in rats (anti-Dcp2 and anti-DcpS) by Covance (Denver, PA). Ascaris anti-eIF4E was prepared against full-length recombinant protein (Lall et al., 2004) in rabbits by Covance.

#### Immunostaining

Young C. elegans adults were dissected on a polylysine-coated slide in M9 buffer (22 mM KH<sub>2</sub>PO<sub>4</sub>, 85 mM NaCl, 1 mM MgSO<sub>4</sub>). Embryos were then "freeze-cracked" (Miller and Shakes, 1995) and fixed in ice-cold MeOH for 10 min. After fixation, embryos were washed in 1× phosphate-buffered saline (PBS; 140 mM NaCl, 2.7 mM KCl, 8 mM Na<sub>2</sub>PO<sub>4</sub>, 2 mM KHO<sub>4</sub>, pH 7.5) and then incubated for 1 h in primary antibody diluted in PTW (1× PBS, 0.05%)



Figure 1. C. elegans Dcp2 is found in cytoplasmic bodies from early embryogenesis. (A) Western blots using the anti-Dcp2 antisera on C. elegans mixed stage embryo extract reveal a dominant band close to 90 kDa. A second lower band was observed upon Western blotting of C. elegans embryonic extracts with two different antisera raised to Dcp2 protein. This lower band may represent a breakdown product of Dcp2, as a similar but slower migrating band is frequently observed upon purification of recombinant Dcp2 (Cohen et al., 2005). (B) dcp2 RNAi depletes the particular staining, indicating that these foci are due to specific staining of Dcp2. Punctate Dcp2 staining is observed in embryos treated with dsluciferase RNA (top panel), but is absent in the majority of embryos treated with dsdcp2 RNA (bottom panel). In addition the disappearance of these

particles upon ds*dcp*<sup>2</sup> treatment suggests that this RNAi treatment diminishes levels of the protein. This shows that the immunostaining is specific and that Dcp2 RNAi is depleting the protein recognized by the antibody. Embryos shown are at the division following the four-cell stage when particles are easily visualized. (C) Immunolocalization of Dcp2 shows that punctate staining is first observed before the pronuclei fuse (top row). Cytoplasmic Dcp2 foci are seen in both the somatic cells and the germ line lineage in the 2-cell (second row), and 8-cell (third row) embryo. The fourth row shows a closer view of punctate Dcp2 staining in an embryonic cell in C. The intensity of foci typically appears to increase during the initial divisions in development up to the 8-cell stage, with strong labeling of somatic foci then observable throughout embryogenesis. This is consistent with an increase in Dcp2 foci as maternal transcripts are turned over or as zygotic transcription initiates. (D) CGH-1 and Dcp2 completely colocalize in cytoplasmic foci in early embryos, with CGH-1 becoming difficult to detect subsequently in development.

Tween-20). After several washes in PTW, embryos were incubated for 1 h in the appropriate secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA), diluted 1: 200 in PTW. After washing in PTW, embryos were mounted in Vectashield (Vector Laboratories, Burlingame, CA). Images were captured using a Leica DMRA2 microscope (Deerfield, IL), Hamamatsu ORCA-ER camera (Bridgewater, NJ), and OpenLab software (Improvision, Lexington, MA). Anti-Dcp2 and -DcpS antibodies were used at a final dilution of 1:200. P-granules were visualized using a mixture of the monoclonal antibodies K76 and OIC1D4, each at a final dilution of 1:10 (Strome and Wood, 1983; Strome, 1986; Miller and Shakes, 1995) and obtained from the Iowa Developmental Studies Hybridoma Bank (http://www.uiowa.edu/ ~dshbwww/index.html). Antibodies to GLH-1 were the kind gift of Karen Bennett and Susan Strome and were used at a final dilution of 1: 200.

### Western Blots

Total *C. elegans* or *Ascaris suum* extract (6 µg with respect to protein) was boiled in Laemmli buffer and then separated by SDS-PAGE on a 4–15% Criterion gel (Bio-Rad). Electroblotting was carried out for 2 h onto PVDF membrane (Bio-Rad). The membrane was washed in H<sub>2</sub>O and then incubated for 30 min in 1% bovine serum albumin in TBST (20 mM Tris, pH 7.5, 150 mM NaCl, 0.05% Tween-20). The blot was incubated for 1 h in primary antibody diluted 1:2500 in TBST buffer. The blot was then washed three times for 5 min in TBST and incubated for 1 h in 1:5000 diluted anti-rat-AP (Sigma, St. Louis, MO). After washing in TBST, the blot was developed for 2–5 min in Western Blue Alkaline phosophatase reagent (Promega, Madison, WI).

#### **Double-stranded RNA-mediated Interference**

Templates for synthesizing double-stranded RNA (dsRNA) were generated by PCR from the appropriate cloned cDNA, using primers containing T7 promoter sequence at their 5' ends. PCR product, 1  $\mu$ g, was used as template in a transcription reaction, using the Megascript in vitro transcription kit (Ambion, Austin, TX). The reaction was incubated for 4 h at 37°C. The quality and quantity of dsRNA was assessed using both electrophoresis and spectrophotometry. dsRNA was precipitated in NaOAc/EtOH, and stored at  $-80^{\circ}$ C for use within 1 wk.

For RNAi, 5  $\mu$ g dsRNA was spun down and the pellet was washed in 75% EtOH. After drying, the dsRNA was resuspended in 4  $\mu$ l soak buffer (3 mM spermidine, 0.05% gelatin in 0.25× Mg<sup>2+</sup>-free M9 buffer, see above) or 5  $\mu$ l M9 buffer (for injection). For soaking, 10–20 *C. elegans* L4 larvae were washed and added in 1  $\mu$ l soak buffer to the resuspended dsRNA. The nematodes were incubated at 20°C overnight and then recovered onto OP50 bacteria-seeded plates overnight. Treated worms were separated onto individual plates, and then allowed to lay eggs for 24 h. At this point adults were removed, and more than 15 h later the number of hatched and unhatched embryos was scored as a measure of embryonic lethality.

For RNAi by injection, young adults were microinjected with dsRNA, and allowed to recover for 6 h. Worms were then allowed to lay eggs for 2  $\,$ 

consecutive 24-h periods, and the embryos were allowed to develop and were scored as described above.

# RESULTS

### C. elegans Dcp2 Is Enriched in Cytoplasmic Bodies

To test the hypothesis that decapping enzymes are found in cytoplasmic foci during metazoan development, we raised polyclonal antibodies to Dcp2. We expressed a fragment of the Dcp2 coding region (residues 1–1430, Cohen *et al.*, 2005), with a Histidine tag, Ni<sup>+2</sup>-NTA agarose purified the tagged protein, and separated a 76-kDa band by SDS-PAGE (which has been shown to have Dcp2 type decapping activity resulting in an m7Gpp product; Cohen *et al.*, 2005). The protein was excised from the gel and used to raise polyclonal antibodies. The resulting sera recognized a band running at 90 kDa in a *C. elegans* mixed stage embryo extract by Western blot (Figure 1A). This is around the expected size of full-length Dcp2 protein (85 kDa).

Immunostaining of C. elegans embryos revealed that Dcp2 protein was found enriched in particles in the embryonic cytoplasm (Figure 1), and these particles disappear in embryos depleted of Dcp2 using RNAi (see below and Figure 1B). Dcp2 staining particles were on average  $0.5 \pm 0.03 \ \mu m$ in size, and many foci were observed per cell. Although it is difficult to count the exact number of foci in a given cell, the number of Dcp2 enriched bodies per cell more closely resembles the many particles observed in mammalian cells than the 2.4  $\pm$  1.4 foci per cell observed in yeast (Sheth and Parker, 2003). Though present around nuclei in the germ cells of the gonad, cytoplasmic (nonperinuclear) Dcp2 foci were first obvious after fertilization and were present by the time the two pronuclei meet and fuse (Figure 1C). By the two-cell stage, Dcp2 bodies were observed throughout the cytoplasm of both the somatic and germ line lineage, though they often appear stronger in the P-cell than the somatic cell at this early point in development (Figure 1C). From the four-cell stage onward, many Dcp2 foci were observed in the cytoplasm of all cells. Dcp2 staining was faint

in nuclei. Because it has previously been noted that CGH-1 (the *C. elegans* ortholog of *S. cerevisiae* Dhh1, an enhancer of decapping) is found in granules throughout the soma during early development, we tested whether CGH-1 and Dcp2 colocalize. We find that somatic CGH-1 foci are coincident with Dcp2, indicating that Dcp2 particles also contain a second homolog of a protein found in *S. cerevisiae* P-bodies (Figure 1D). In contrast to previous observations showing that CGH-1 somatic foci disappear after the first few embryonic divisions, we observed that cytoplasmic Dcp2 foci persist throughout embryonic development.

# C. elegans *Dcp2* Is Found in P-Granules in a pgl-1dependent Manner

P-bodies have been previously compared with C. elegans P-granules because they both contain proteins associated with RNA regulation. CGH-1 is a C. elegans helicase whose yeast ortholog is known to enhance decapping activity. However, CGH-1 has not been specifically demonstrated to play a role in C. elegans RNA decay and it is not known if P-granules are enriched in other components of mRNA degradation machinery. Transcripts as well as proteins involved in mRNA translation have been found to colocalize with P-granules, suggesting a role in translational regulation (Amiri et al., 2001; Hanazawa et al., 2004). We observed by immunostaining that the germ line precursor cells contained much larger sites of Dcp2 localization that resembled Pgranules in shape. To test the hypothesis that Dcp2 is found in P-granules, we colabeled Dcp2 immunostained C. elegans embryos with monoclonal antibodies that stain P-granules (K76 and OIC1D4) and also with anti-GLH-1, which has previously been shown to label P-granules (Gruidl et al., 1996). Such immunostaining indicates that Dcp2 is enriched in P-granules (Figure 2A). Dcp2 is also present at sites where P-granules are observed in C. elegans germ cells during oogenesis (see Figure 5A).

To test whether the localization of Dcp2 is dependent on components that affect P-granules, we immunostained pgl-1 (ct131, bn101, and bn102) and glh-1 (ok439 as well as gk100) mutants with the Dcp2 antibody. Although Dcp2 is still observed in some P-granules in the pgl-1 (ct131, bn101, and bn102) mutants at the restrictive temperature, this localization is reduced when compared with wild-type embryos (Figure 2B). In contrast GLH-1 continued to be associated with P-granules in the pgl-1 (ct131 as well as bn101, and bn102) mutants, consistent with previous observations in gonads (Figure 2B; Kawasaki et al., 2004). Somatic Dcp2 bodies continue to be observed in *pgl-1* mutant (Figure 2). This is not the case in *glh-1* (*ok439* as well as *gk100*) mutants, where Dcp2 immunolabeling in the P cell appears relatively normal (unpublished data). This was found to be the case in three *pgl-1* strains carrying temperature-sensitive mutations (alleles ct131, bn101, and bn102, which lead to 75-85% sterility at the restrictive temperature; Kawasaki et al., 1998), and two glh-1 deletion mutants (deletion alleles ok439 and gk100, which affect different regions of the glh-1 gene and were obtained from the International C. elegans gene knockout consortium). In summary, Dcp2 is found in P-granules, showing that a component of the mRNA degradation machinery localizes to these sites in a manner that is compromised in *pgl-1* mutants.

# C. elegans DcpS Is Found throughout the Cytoplasm

In both mammalian cells and yeast, there are two major and general pathways by which mRNA degradation occurs. We tested whether DcpS, the enzyme involved in cap metabolism after exosome-mediated transcript degradation, is also



**Figure 2.** Staining with multiple markers indicates that Dcp2 colocalizes with P-granules. (A) Dcp2 immunolocalization was carried out along with the K76/OICD14 antigen (top row, right panel), or GLH-1 (bottom row, right panel). Dcp2 is seen to colocalize with these markers in P-granules. (B) Dcp2 localization to P-granules is diminished in *pgl-1* (*bn101*) mutants. L4 larvae were moved to the restrictive temperature (25°C) until adulthood, after which embryos were stained with the anti-Dcp2 and anti-GLH-1 antibodies. Shown are close ups of the P-cell at the 16-cell stage. Although wild-type (N2) embryo P-granules (marked by GLH-1) contain Dcp2, the levels of Dcp2 are diminished in P-granules of *pgl-1* mutants (bottom panels).

found at specific cytoplasmic sites. DcpS has previously been localized to mammalian cell nuclei, though it is involved in the dominant pathway of cytoplasmic mRNA degradation in mammals (Wang and Kiledjian, 2001; Liu et al., 2002; Cougot et al., 2004a). We raised polyclonal antibodies to the entire coding region of *C. elegans* DcpS (described in Cohen *et al.,* 2004). The resulting sera recognized a dominant band of 32 kDa in C. elegans embryo extracts by Western blot, just below the predicted size of DcpS (36.5 kDa; Figure 3A). This is also consistent with the size of the recombinant protein that can recapitulate DcpS activity in vitro. By immunostaining C. elegans embryos we found that there was a broad distribution of DcpS throughout the cytoplasm of all lineages (Figure 3B). This labeling disappears in the majority (80%) of dsdcpS RNA-treated embryos. DcpS was not generally observed in cytoplasmic bodies, although we infrequently observed a region of more intense DcpS



**Figure 3.** *C. elegans* DcpS is found throughout the cytoplasm during embryogenesis. (A) A Western blot using the polyclonal anti-DcpS sera shows a dominant band in *C. elegans* mixed stage embryo extract of ~32 kDa. (B) Immunolocalization of DcpS in embryos shows signal throughout the cytoplasm just after fertilization (top row), with some intense staining observed in the majority of embryos around the male pronucleus. In older embryos, DcpS is observed throughout the cyto-

plasm, although sites containing higher levels of signal are observed, albeit infrequently (arrows). (C) d*sluciferase* RNA does not affect DcpS immunostaining levels (top panel), whereas ds*dcpS* treatment does (bottom), indicating that the antibody is specifically labeling DcpS and that RNAi is effective at reducing protein levels.

labeling in the cytoplasm of some cells. These regions of more intense staining are not observed in every embryo and are not enriched at a particular stage of development, although they may be unstable under fixation conditions tested (arrow, Figure 3B). Though it has previously been shown that mammalian DcpS is found in the nucleus, our observation of significant cytoplasmic DcpS in C. elegans is consistent with previous biochemical evidence from the nematode A. suum. In particular, the predominantly cytoplasmic location of DcpS is consistent with measurements of DcpS decapping activity in subcellular fractionation experiments using nematode A. suum embryo extracts (Cohen et al., 2004). Our immunostaining allows us to conclude that the bulk of DcpS signal is observed cytoplasmically, a situation consistent with S. cerevisiae (Malys et al., 2004) and that enzymes involved in mRNA cap metabolism are not all enriched in cytoplasmic bodies. Therefore although some DcpS may be present in C. elegans nuclei, we do not observe the predominantly nuclear localization suggested in other systems (Salehi et al., 2002; Cougot et al., 2004a; Liu et al., 2004).

# Dcp2 and DcpS RNAi Indicate that Decapping Proteins Are Not Individually Required for Embryonic Development

Having found that the decapping enzymes, Dcp2 and DcpS, are expressed during *C. elegans* embryogenesis, we next tested whether these enzymes play a critical role in devel-

opment. We used dsRNA-mediated interference (RNAi) to decrease function of *dcpS*, *dcp2*, and its partner *dcp1*. Although we introduced dsRNA by different methods (soaking and injection), carried out the experiments in wild-type as well as an RNAi-sensitive strain (rrf-3), and targeted different regions of *dcp2*, RNAi for any one of these three genes does not compromise embryonic development in an obvious manner (Table 1). No phenotype was observed in postembryonic stages of development, and the F1 progeny of treated worms were not sterile. The level of embryonic lethality observed by RNAi targeting *dcp1*, *dcp2*, or *dcpS* was not much greater than during treatment with a negative control (dsluciferase RNA). This was true by treatment at two different temperatures, (20 vs. 25°C, unpublished data) and was also the case at different time points after treatment (embryos laid 6-24, 12-24, and 24-48 h after RNAi treatment of the parents). We also tried targeting two regions of dcp2 in independent RNAi experiments but saw little embryonic lethality (Table 1). This suggests that lack of phenotype was not due to the particular sequence targeted during RNAi. Time-lapse movies of the early development of RNAi-treated embryos also appeared normal (unpublished data), suggesting that there is not a subtle effect due to RNAi that embryos subsequently recover from later in development. To test whether RNAi was effectively depleting the targeted protein, we immunostained embryos from treated

Table 1. Effect of reducir	g decapping enzyme	levels by RNA interference	on embryogenesis
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DsRNA	Region of transcript targeted	Mean % embryonic lethality in wild typeª	Mean % embryonic lethality in <i>rrf-3</i> mutant background
dcp2 (Y45F10A.1)	1–969	5.0 (0–10; n = 77)	10.8 (0-27.2)
dcp2	1731-2480	5.2(0-9.6; n = 20)	ND <sup>b</sup>
dcp1 (Y55F3AM.12)	42-995	1.4(0-4; n = 62)	9.8 (0-17.3)
dcpS (Y113G7A.9)	9–919	3.7(0-5.3; n = 54)	17.8 (0-42.3)
<i>ife-2</i> and <i>ife-5</i> (R04A9.4 and Y57A10A30)	367–687 and 621–833	15.5 (0-100; n = 20)	ND
<i>dcp1</i> and <i>dcp2</i>	As above	20.2 (0-38.1; n = 38)	12.8 (0-21.4)
<i>dcp2</i> and <i>dcpS</i>	As above	9.0 $(0-23; n = 35)$	21.2 (5.8–59.5)
Negative control	1–981	3.7 (0-5.5; n = 49)	8.4 (0–14.2)
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The negative control consisted of  $ds\beta$ -gal RNA or dsluciferase RNA treatment. For dsdcp2 and dsdcpS RNA treatments, progeny of treated worms were immunostained to check the effectiveness of RNAi-mediated depletion: Dcp2 foci were gone in all embryos checked, and DcpS staining was decreased in the majority of embryos examined, with some residual staining evident in a percentage of embryos (20%), though this percentage does not account for the low levels of embryonic lethality observed. Treatments were carried out on at least four independent occasions. RNAi for dcp2 was carried out independently using two different regions of the gene. Data shown are for progeny laid 24–48 h after treatment with dsRNA, but higher embryonic lethality rates were not observed in embryos laid 12–24 or 48–72 h (unpublished data). The experiments were also carried out in an *rrf-3* mutant (right column), a mutant that is more susceptible to RNAi.

<sup>a</sup> The range of embryonic lethality observed is given in parentheses adjacent to the mean percentage embryonic lethality, with 0% indicating that all progeny hatched. n, number of dsRNA treated hermaphrodites, their progeny being assessed for embryonic lethality. <sup>b</sup> ND, experiment not done.

dsRNA	Putative activity of target	Region of transcript targeted	Mean % embryonic lethality <sup>a</sup>
xrn-1 (Y39G8C.1)	5'-3' exonuclease	1547-2220	62.0 (44.4–88.8; n = 41)
eIF4G (M110.4)	Translation initiation factor	16-1064	$61.2 (0-100; n = 15)^{b}$
ife-3 (B0348.6c)	Translation initiation factor	396-762	95.1(56-100; n = 20)
ife-4 (C05D9.5)	Translation initiation factor	408-639	11.8(7-16; n = 20)
pab-1(Y106G6H.2)	Poly (A) tail-binding protein	1251-1736	$100 (n = 15)^{b}$
cbp-20 (F26A3.2)	Nuclear cap-binding protein subunit	1–463	98.7(94.9-100; n = 45)
cbp-80 (F37E3.1)	Nuclear cap-binding protein subunit	2–1120	63(36.4-100; n = 20)

Table 2. Effect of reducing activity of factors involved in mRNA function and turnover by RNA interference

Embryonic lethality was scored as described in *Materials and Methods*, scoring embryos laid 24–48 h after dsRNA treatment.

<sup>a</sup> The range of embryonic lethality observed is given in parentheses adjacent to the mean percentage embryonic lethality. 0% indicates that all laid progeny hatched, i.e., no embryonic lethality was observed. n, number of dsRNA treated hermaphrodites, their progeny being assessed for embryonic lethality.

<sup>b</sup> dsRNA treatments that led to reduced fecundity (average brood size =  $2.4 \pm 2.6$  per worm for dspab-1 RNA treatment and 7.7  $\pm 1$  1.2 for dseIF4G experiments).

mothers with the Dcp2 and DcpS antibodies. We find that both Dcp2 and DcpS appear largely depleted in all RNAitreated embryos (Figures 1B and 3C), an effect not seen in dsluciferase RNA-treated embryos (Figures 1B and 3C). We favor the hypothesis that Dcp2 is being depleted at its site of action as it is known that *S. cerevisiae* dcp1, dcp2, and dcpS mutants are viable (Beelman et al., 1996; Dunckley and Parker, 1999; Liu et al., 2002). Although we found that Dcp2 is localized to P-granules, the progeny of dcp2 RNAi-treated mothers did not show sterility (unpublished data), showing that depletion of Dcp2 alone from the P-granules does not affect germ line development. In addition P-granules appeared normal in dsdcp2-treated embryos, as visualized by the anti-GLH-1 antibody (unpublished data).

The lack of effect upon depletion of *dcp1*, *dcp2*, or *dcpS* alone, led us to hypothesize that there may be redundancy in the two major decay pathways compensating for RNAi treatment. We therefore tested whether depletion of multiple decapping factors by RNAi affects embryonic development. On RNAi-based depletion of both *dcp1* and *dcp2*, we saw a small increase in embryonic lethality. Indeed, the mean percentage of embryonic lethality increased from 5.2 to 20.2% upon double RNAi (Table 1). Lethality in the double dcp1/dcp2 RNAi treatment is significantly different from the negative control (p = 0.02, test examining the hypothesis that the *dcp1/dcp2* RNAi is different from the negative control). The statistical significance of this increase relative to the single *dcp2* (RNAi) treatment is low, however, due to the large degree of variation in the two data sets, as evident in the range of lethality observed in Table 1 (p = 0.25, test examining whether the data arising from *dcp2* and *dcp1/dcp2* RNAi are different). Though this increase is low, such an increase in lethality upon dsdcp1/dsdcp2 RNAi treatment is consistent with data, suggesting that Dcp1 may enhance the activity of Dcp2 in yeast (Steiger et al., 2003). Our data are thus also consistent with a model where depletion of both proteins more effectively disrupts the activity of a decapping "holoenzyme." Simultaneous treatment of *C. elegans* with dsdcp2 and dsdcpS led to a small, but insignificant, increase in embryonic lethality (increased to 9% on average; Table 1). In cases of embryonic lethality, embryos died at various points late in development. Immunostaining revealed that there was at least some residual DcpS present in double RNAi-treated embryos (unpublished data). Thus the low phenotypic penetrance seen in double RNAi-treated embryos may be related in part to the documented inefficiency of double RNAi (see for example, Parrish et al., 2000). To test

whether (as in yeast) depleting an exosome component along with either *dcp1* or *dcp2* is lethal, we carried out double RNAi for the *C. elegans* homolog of the cytoplasmic exosome component, Ski2. The most likely homolog of *ski2* is *C. elegans* F01G4.3 (Blast Score 3e-212, when compared with *H. sapiens ski-2*). RNAi by soaking or injection did not lead to significant lethality for *dcp2/ski2* or *dcp1/ski2* depletions (unpublished data). This could be due to insufficiently decreased levels of *ski2*, but it should also be noted that there is a very similar gene in the *C. elegans* genome W08D2.7 (49% identity at the amino acid level), so that W08D2.7 may compensate for *F01G4.3* depletion. Indeed mRNA decay in *C. elegans* may be more complicated than the processes defined in yeast, and involve multiple redundant components and pathways.

We further tested whether the lack of high penetrance phenotypes was due to inefficiency of RNAi by repeating these experiments in the rrf-3 mutant (Simmer et al., 2002). This mutant shows increased sensitivity to dsRNA. We observed an increase in embryonic lethality in the progeny of dsdcpS, dsdcp2, and dsdcp2/dsdcpS-treated animals, but did not see a highly penetrant lethality in the context of rrf-3 mutants (Table 1). Although residual protein may still be present, this shows that increased sensitivity to RNAi (in the context of the rrf-3 mutant) does not reveal an absolute requirement for decapping proteins in our RNAi experiments. Although Xrn-1 acts downstream of Dcp2 in yeast and mammalian mRNA decay, depletion of C. elegans xrn-1 leads to lethality at a specific stage in development (ventral epithelial closure, Table 2) that is not observed for Dcp1 or Dcp2. This suggests that Xrn-1 may play a specific role in C. elegans development that is Dcp1/Dcp2 independent.

The incomplete phenotypic penetrance of dcp1/dcp2 double RNAi may be due to the inefficiency of double RNAi, even in the context of the *rrf-3* mutant, but may also be due to functional redundancy with other mRNA degradation pathways. To further test for interactions between mRNA decay pathway genes we examined the effect of reducing function of dcp1, dcpS, and xrn-1 on Dcp2 foci. Reducing function of these genes has little quantifiable effect on the foci in embryos: in general they appear to be present in the same size and number (Figure 4). However depleting levels of an upstream enzyme in the mRNA degradation pathways does affect Dcp2 localization. In particular RNAi targeting ccf-1 (the homolog of *S. cerevisiae* SGD:YNR052C, an RNase of the CCR-4-NOT associated yeast deadenylation complex), leads to decreased Dcp2 foci in embryos (Figure 4). In ad-



**Figure 4.** Effect of dsRNA treatments on Dcp2 foci. Treatment with ds*dcp-1*, ds*xrn-1*, and ds*dcpS* RNA do not affect Dcp2 focal staining in a consistent manner. However *ccf-1* RNAi leads to a misshapen embryo and multinucleate cell (unpublished data), as well as a marked decrease in Dcp2 staining in embryos examined, an effect that is not seen in the context of other RNAi treatments, including that with ds*luciferase* RNA (Figure 1B). Because the number and density of foci per cell is difficult to quantify and varies between cells and focal planes, it is difficult to verify or claim changes in foci number in these treatments.

dition RNAi targeting *cgh-1* leads to highly penetrant sterility in treated *C. elegans* (100% of treated animals) and a concomitant affect on Dcp2 foci in the germ line (Figure 5). In this case, some Dcp2 staining in *cgh-1*(RNAi) gonads have more intense general cytoplasmic Dcp2 staining, although perinuclear foci are still observed (Figure 5). The subtlety of these effects is not surprising given that *dcp2* RNAi and *cgh-1* RNAi have such different phenotypes (no phenotype as opposed to highly penetrant sterility). Furthermore nuclei that appear abnormal by DAPI staining have disrupted



**Figure 5.** *cgh-1* RNAi affects Dcp2 staining in the germline. Dcp2 is observed in punctate perinuclear structures as well as generally in the gonadal cytoplasm in  $\beta$ -gal (RNAi)-treated gonads (top panels, Dcp2 shown on right, DAPI on left). Although treatment with ds*gh-1* leads to a highly penetrant sterile phenotype, which means foci cannot be assessed in early embryos, Dcp2 staining in the gonad is subtly altered in this RNAi treatment. Typically perinuclear staining remains, but more signal is observed in the general cytoplasm of worms treated with ds*cgh-1* RNA. Furthermore, abnormal looking nuclei (as assessed by DAPI staining) are not surrounded by Dcp2 (see arrowhead).

Dcp2 staining, with few cytoplasmic foci visible (arrowhead in Figure 5). Thus RNAi for at least two genes whose orthologues have been associated with mRNA decay pathways in other species (*ccf-1* and *cgh-1*) lead to changes in Dcp2 localization in *C. elegans*, with only subtle changes observed in *cgh-1* RNAi treatments.

# Other Genes Involved in mRNA Cap Interaction Are Critical for Development

We have previously described a component of the nematode cap recognition complex, eIF4G (Lall et al., 2004). This protein interacts with eIF4E and acts as a scaffold for interactions with other translation factors (Gingras et al., 1999). Indeed we have previously shown that this C. elegans eIF4G ortholog has conserved activity, increasing the efficiency of eIF4E/mRNA interaction in vitro. RNAi of eIF4G in C. elegans leads to highly penetrant embryonic lethality (on average 61% embryos do not hatch; Table 2). Thus eIF4G is absolutely required for C. elegans development. The brood size of dseIF4G-treated worms is reduced substantially (a mean of 7.7  $\pm$  11.2 embryos laid per worm for dselF4Gtreated worms, as opposed to  $48.1 \pm 20.9$  embryos per worm for dsluciferase-treated worms). Thus lack of eIF4G leads to sterility in the treated worm. This reduced fecundity is similar to that observed in a previous study, though the degree of embryonic lethality is considerably more severe than the phenotypes noted previously (Long et al., 2002). Poly(A)binding protein (PAB-1) is a factor that binds the poly(A) tail of mRNAs and has previously been shown to interact with the 5' end of the mRNA by binding to eIF4G (Coller and Parker, 2004; Kuhn and Wahle, 2004). pab1 RNAi leads to a very high level of embryonic lethality (100%) and also leads to sterility, as reflected in decreased brood size (2.4  $\pm$  2.6 embryos per worm). eIF4G and PAB-1, which interact with one another to promote translation efficiency, are therefore critical for fecundity as well as embryonic development.

We also examined the effect of down-regulating components of the nuclear cap-binding complex, CBP-20 and CBP-80. The latter gene has recently been genetically linked to mRNA decay in a study in S. cerevisiae (Das et al., 2003). The nuclear cap-binding complex components are found to be essential for development, leading to 63% (CBP-80), and 98.7% (CBP-20) embryonic lethality in the offspring of treated worms. This is in contrast to S. cerevisiae, where strains mutant for the nuclear cap binding complex proteins are viable (Uemura and Jigami, 1992; Colot et al., 1996; Giaever et al., 2002). The brood size of worms treated with dscbp-20 and dscbp-80, appear relatively normal (a mean of 56 and 27 embryos laid per worm, respectively), so that RNAi for these genes does not affect C. elegans fecundity. Thus a spectrum of phenotypes from no effect to embryonic lethality and sterility is observed upon RNAi treatment of C. elegans cap-interacting factors.

### Localization of CBP-20 during C. elegans Development

We also ascertained the localization of CBP-20 using human antibodies that are cross-reactive. The human CBP-20 antibody recognizes a nuclear enriched protein in *C. elegans* (Figure 6A). In *C. elegans* embryos staining of CBP-20 is strikingly strong at the nuclear envelope (arrow, Figure 6A), though some diffuse as well as punctate cytoplasmic staining is also observed. Depletion of *cbp-20* by RNAi (against gene F26A3.2) led to loss of this nuclear periphery staining (Figure 6B). The human CBP-20 antibody also gives a nuclear signal upon immunostaining of *A. suum* embryos (unpublished data), but does not recognize a band on a Western blot on nematode (*A. suum*) embryo extract. Thus it is for-



**Figure 6.** Immunolocalization of CBP-20 in *C. elegans* embryos. (A) Anti-CBP-20 immunostaining reveals localization at the nuclear envelope (staining at nuclear periphery indicated by arrow). (B) This localization is not observed in embryos treated with dscbp-20 RNA, indicating that the antibody is likely recognizing CBP-20. (C) Immunostaining with a cross-reactive antibody raised to *A. suum* (nematode) eIFE-3 shows general cytoplasmic staining in *C. elegans* embryos, rather than overt staining of cytoplasmic foci. The arrow indicates P-granule staining by the anti-eIF4E antisera.

mally possible that CBP-20 immunostaining is due to recognition of another conserved protein containing similar motifs (RRM, or RNA-binding motifs). However the sequence of dsRNA used, although encompassing most of the *cbp-20* open reading frame, does not show sufficient similarity at the nucleotide level to be predicted to mediate off-target depletion of other genes in the C. elegans genome (www.rnai.org). The loss of signal upon cbp20 (RNAi) therefore indicates that the antibody, raised to the human CBP-20 antigen, is likely recognizing CBP-20 protein. The peripheral nuclear staining is consistent with CBP-20 accompanying newly synthesized mRNAs to at least the nuclear envelope. In addition it has recently been found that the translation initiation factor eIF4E is found in cytoplasmic foci in mammalian cells (Kedersha and Anderson, 2002; Andrei et al., 2005; Kedersha et al., 2005). To test whether this is the case in C. elegans, we immunostained embryos with an antibody raised to the A. suum eIF4E-3 protein (Lall et al., 2004). We did not observe obvious somatic cytoplasmic foci with this antibody (Figure 6C). Although we cannot eliminate the possibility that this cross-reactive antisera is recognizing a generally localized eIF4E isoform that is masking localization of one isoform in cytoplasmic foci, these data do show that the C. elegans eIF4E proteins are not entirely localized to cytoplasmic foci. In addition, we observe signal in P-granules (Figure 6C, arrowhead). This localization is expected of at least the eIF4E isoform IFE-1 (Amiri et al., 2001). Hence although C. elegans eIF4E signal is observed in P-granules, very general cytoplasmic localization is also observed in all embryos examined. Thus neither eIF4E nor CBP-20 are observed to be localized to foci to the degree that Dcp2 is.

### DISCUSSION

We have shown that Dcp2 localizes to distinct cytoplasmic foci during early C. elegans embryogenesis. In addition Dcp2 is also found in P-granules, colocalizing with the P-granule markers GLH-1 and the antigens recognized by the K76 and OIC1D4 antisera. In contrast the decapping enzyme of the 3' to 5' decay pathway, DcpS is more generally localized throughout the cytoplasm. We have also found that depleting dcp1, dcp2, or dcpS does not affect the viability of the embryo, in contrast to depletion of the endonuclease xrn-1. Our data does, however, suggest an interaction between some decapping factors, as indicated by a modest increase in embryonic lethality during double RNAi targeting *dcp1* and *dcp2*. Several other factors that are either cap-interacting proteins or interact with cap-binding proteins are absolutely required for development, given that RNAi leads to sterility in the treated worm or death of its progeny.

# Cytoplasmic Organization of mRNA Translation and Decay

Our observation that Dcp2 is found in foci leads to the question of whether these are sites of mRNA decay. Dcp2 foci are obvious around the nuclei of oocytes, but become difficult to detect in late oogenesis. Although it is possible that these bodies are sites of Dcp2 storage, strong labeling in the cytoplasm shortly after the onset of embryonic development, as well as somatic foci becoming obvious around the onset of zygotic transcription and at a time when there is turnover of maternal transcripts, suggests that Dcp2 foci may be functional sites. Furthermore, the fact that Dcp2 foci are linked with mRNA degradation in yeast and mammalian cells (Sheth and Parker, 2003; Cougot et al., 2004a) leads us to propose that at least some Dcp2 mediated processing of mRNAs occurs at specific cytoplasmic sites during C. elegans development. Because cytoplasmic Dcp2 foci become very evident at the four-cell stage when there is some embryonic transcription we favor the hypothesis that Dcp2 foci are active sites of decay rather than storage sites (Edgar et al., 1994). In addition, our previous data indicate that Dcp2 type decapping activity is actually strongest in the 130S fraction from fractionated A. suum extracts (Cohen et al., 2004). Although we do not know where P-bodies would sediment during such purification, the 130S fraction is where polysomes are found, suggesting that Dcp2 activity is enriched with multisubunit machinery. This suggests that in nematodes, Dcp2-like activity is strongest in a fraction that may possibly contain structures such as degradation foci. Further evidence supporting the contention that Dcp2 foci represent sites of decapping activity rather than storage comes from our observation that Dcp2 localization at foci is affected by depletion of cgh-1, as well as the deadenylase subunit homolog, ccf-1. In the former case, cgh-1 is a homolog of an enhancer of decapping in yeast. Indeed the *dhh-1* deletion in yeast leads to a decrease in levels of P-body foci. This is consistent with the decreased Dcp2 localization that we observe in the *C. elegans* gonad upon *cgh-1* depletion, though we cannot follow the effect of cgh-1 RNAi on Dcp2 foci in embryos due to the sterile phenotype caused by this treatment. Similarly, depletion of the deadenylation enzymes that act upstream of Dcp1 and 2 in yeast, decreases P-body size and number in yeast (Sheth and Parker, 2003). This is similar to the effect that we see in *C. elegans* upon depletion of the homolog of the S. cerevisiae deadenylase subunit, ccf-1. Interestingly, components of the RNA interference (RNAi) effector complex, RISC, have recently been localized to Pbodies in mammalian cells (Ding *et al.*, 2005; Liu *et al.*, 2005; Sen and Blau, 2005).

In contrast to Dcp2 localization the DcpS-decapping protein is found throughout the cytoplasm. This implies that mRNA degradation is not necessarily confined to Dcp2 foci, given that the decapping enzyme of the 3' to 5' decay pathway is more widely dispersed. Indeed evidence from S. *cerevisiae* shows that 3' to 5' decay pathways components, including DcpS, are not found in foci in this yeast (see http://yeastgfp.ucsf.edu and Malys et al., 2004). Thus although the 5' to 3' decay pathway may be highly organized at the cell biological level, general exosome mediated degradation and DcpS cap digestion seem to be less so. These distinct localizations of Dcp2 and DcpS suggest that different decapping pathways may operate in distinct subcellular locations, albeit with some overlap. This is entirely consistent with crude subcellular fractionation of Ascaris embryos followed by assessment of where DcpS- and Dcp2-like activity are found (Cohen et al., 2004). Such differences in Dcp2

and DcpS localization may be due to the possibility that Dcp2 is involved in regulated degradation of mRNAs that are actively targeted for rapid degradation and must be quickly removed from the vicinity of the translational machinery, although 3' to 5' decay may be a more general pathway for transcript turnover. Although DcpS has previously been detected in the nucleus of yeast and mammalian cells (Salehi et al., 2002; Cougot et al., 2004a; Liu et al., 2004) and Dcp2 can decap trimethylated capped RNAs across species (Cohen et al., 2005) and might be predicted to be involved in decapping snRNAs, the bulk of the immunostaining we observe is cytoplasmic. This does not preclude the presence of significant levels of both of these enzymes in the *C. elegans* nucleus. The fact that a large percentage of *C*. elegans mRNAs are trimethylated in addition to snRNAs and some snoRNAs might explain why the vast majority of detectable decapping protein is observed in the cytoplasm in C. elegans and does not exclude the possibility that functional levels of nuclear Dcp2 and DcpS are present, but difficult to detect over cytoplasmic signal.

# Localization of Dcp2 to P-Granules

Our data indicates that Dcp2 protein is also found in C. elegans P-granules. The role that Dcp2 might have in Pgranules is not entirely clear: although RNA binding proteins including IFE-1 (eIF4E isoform 1), poly(A) polymerase, Nanos, and the GLH helicases localize to P-granules, these are thought to regulate translation. Current models suggest that upon cessation of translation, Dcp1/Dcp2 may gain access to the transcript cap initiating mRNA degradation (Schwartz and Parker, 2000; recently reviewed in Wilusz and Wilusz, 2004 and). Thus the RNAs within P-granules may be subject to this interplay between translational incompetence and consequent decay. The notion that there is active mRNA turnover occurring in P-granules might be strengthened by the observation that the C. elegans ortholog of Dhh1, an enhancer of *S. cerevisiae* Dcp1/Dcp2 decapping, has been localized to P-granules (Navarro et al., 2001). However functional conservation of the activity of this Dhh-1 related protein (CGH-1) has not been demonstrated. Indeed, the proposed function of CGH-1 is thought to be in translational silencing rather than mRNA decay. However it should be noted that P-granules may contain RNAs that have a cap and spliced leader 1 (though the detected TMG cap-SL could be the spliced leader RNA itself, rather than mRNAs), and are also poly(A) positive (Seydoux and Fire, 1994). Alternatively, it may be that Dcp2 localized to Pgranules is sequestered and inactive, promoting the stability of some mRNAs in the P-cell.

# Requirements for Cap Interaction Enzymes during Development

The data presented indicates that, as in yeast, the decapping enzymes are not on their own essential for development. In more detail, it has previously been shown that yeast mutants carrying deletions in *dcp1*, *dcp2*, or *dcpS* are viable. Indeed although degradation of *S. cerevisiae* transcripts such as PGK1 is kinetically affected in *dcp2*-deleted *S. cerevisiae* strains, this mRNA ultimately continues to be degraded, leaving intermediates consistent with exosome mediated decay (Muhlrad *et al.*, 1995; Beelman *et al.*, 1996). These data indicate that there is at least some redundancy between transcript degradation pathways. Thus the lack of phenotype that we observe upon depletion of individual decapping proteins in *C. elegans* is entirely consistent with the observation that these proteins are dispensable in yeast. The fact that the exosome can degrade mRNAs in the absence of DcpS suggests that one would not expect a phenotype arising from the stabilization of mRNAs in the context of double RNAi targeting *dcp2* and *dcpS*. However the lack of a phenotype in the double RNAi for *dcpS* and *dcp2* suggests either 1) there may be other pathways for cap turnover, 2) that lack of cap-degradation is not toxic or lethal, or 3) that there is a low level of residual activity in the RNAi experiments that masks the latter effect.

This is in contrast to C. elegans xrn-1, RNAi of which results in failure of ventral epithelial closure (Newbury and Woollard, 2004). This suggests that xrn-1 may have specific and essential roles that are independent of Dcp1 and 2 during embryogenesis or that there are other unknown enzymes that act redundantly to decap full-length mRNAs in the 5' to 3' decay pathway during embryogenesis. Such an analysis is also applicable to cgh-1, RNAi of which leads to sterility. Once again this suggests that *cgh-1* either has roles during gonadogenesis that are independent of *dcp2* or that unknown decapping factors act redundantly with Dcp2 to allow CGH-1 to carry out its function. In this case however, cgh-1 is a potential ortholog of ste13/ME31B/RCK/p54, putative RNA helicases involved in reproduction (Navarro et al., 2001) and may therefore be predicted to act independently of Dcp2 in the germ line. Thus although CGH-1 is localized to the same cytoplasmic bodies as Dcp2, it may have a distinct role. One possibility is that CGH-1 plays an indirect role in mRNA decay. If, for example, CGH-1 was involved in translational control, it may appear to be localized to "P-bodies" and act as a promoter of decapping in yeast, even if it does not participate directly in mRNA decay. Such a model could similarly apply to deadenylases such as CCF-1. The adverse effect of deadenylation on translation may indirectly induce the onset of decapping. This would lead to the conclusion that enzymes involved in distinct but linked processes (translational control and decay) are colocalized within single, multifunctional, cytoplasmic bodies (both P-bodies and P-granules). This would explain why the phenotypes of cgh-1 and dcp2 are so distinct and also why CGH-1 has a distinct temporal pattern of cytoplasmic localization in the C. elegans embryo, appearing to leave Dcp2enriched foci during early development.

The lack of lethality seen in RNAi targeting the decapping proteins seems surprising given that methylated cap structures inhibit translation in vitro and might be misincorporated into nucleic acids (Cougot *et al.*, 2004b; Lall *et al.*, 2004). Therefore, they are expected to be poisonous if they accumulate in vivo upon depletion of Dcp2 and Dcp5. This would especially be the case if DcpS action were a common final step in both degradation pathways, as has recently been suggested (van Dijk *et al.*, 2003). However our previous data indicates that nematode DcpS does not turnover the product of Dcp1/2-mediated cap digestion (Cohen *et al.*, 2004).

Finally our data indicate that eIF4G, PAB-1, CBP-20, and CBP-80 are absolutely essential for development. This contrasts with some of the eIF4E cap-interacting translation initiation factors. The eIF4E genes are known to have duplicated and specialized in many organisms. This is indicated in *C. elegans* by the differential cap binding properties, as well as specialized developmental functions of the IFE isoforms (Keiper *et al.*, 2000; Amiri *et al.*, 2001; Dinkova *et al.*, 2005). However it seems that the nuclear cap-binding proteins have remained absolutely essential for development in *C. elegans*. In addition, nematode DcpS has adapted to hydrolyze both trimethylated and monomethylated mRNA caps and Dcp2 in yeast, mammals, and nematodes exhibit dual cap specificity (Cohen *et al.*, 2004, 2005). Thus the presence of trimethylated and monomethylated mRNA caps does not necessarily necessitate an increase in isoform number of cap-interacting proteins. Interestingly both eIF4G and PAB-1 are also essential for development. Thus some proteins involved in mRNA metabolism seem dispensable for development (such as the decapping proteins in decay, and certain IFE isoforms in translation), although proteins functioning in the very same process (such as Xrn-1 in decay, and eIF4G in translation initiation) are critical during development in *C. elegans*.

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