Developmental Biology 322 (2008) 276–288

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/developmentalbiology

Drosophila processing bodies in oogenesis

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ARTICLE INFO

Article history:
Received for publication 27 February 2008
Revised 14 July 2008
Accepted 15 July 2008
Available online 5 August 2008

Keywords:
Processing bodies
Drosophila decapping protein 1
Drosophila decapping protein 2
Pacman
Me31B
Oogenesis

ABSTRACT

Processing bodies (P-bodies) have emerged as important subcellular structures that are involved in mRNA metabolism. To date, a detailed description of P-bodies in *Drosophila* oogenesis is lacking. To this end, we first demonstrate that *Drosophila* decapping protein 2 (dDcp2) contains intrinsic decapping activity and its enzymatic activity was not detectably enhanced by *Drosophila* decapping protein 1 (dDcp1). dDcp1-containing bodies in the nurse cell cytoplasm can associate with the 5′ to 3′ exoribonuclease, Pacman in addition to dDcp2 and Me31B. The size and number of dDcp1 bodies are dynamic and dramatically increased in *dDcp2* and *pacman* mutant backgrounds supporting the conclusion that dDcp1 bodies in nurse cell cytoplasm are *Drosophila* P-bodies. In stage 2–6 oocytes, dDcp1 bodies appear to be distinct from previously characterized P-bodies since they are insensitive to cycloheximide and RNase A treatments. Curiously, dDcp2 and Pacman do not colocalize with dDcp1 at the posterior end of the oocyte in stage 9–10 oocytes. This suggests that dDcp1 bodies are in a developmentally distinct state separate from the 5′ end mRNA degradation enzymes at later stages in the oocyte. Interestingly, re-formation of maternally expressed dDcp1 with dDcp2 and Pacman was observed in early embryogenesis. With respect to developmental switching, the maternal dDcp1 is proposed to serve as a marker for the re-formation of P-bodies in early embryos. This also suggests that a regulated conversion occurs between maternal RNA granules and P-bodies from oogenesis to embryogenesis.

Introduction

Tight regulation of gene expression is critical for the success of developmental processes. Recently, the role of messenger RNA degradation has emerged as an important step in the regulation of gene expression (reviewed in Garneau et al., 2007). The impairment of the 5′ to 3′ mRNA degradation pathway can result in detrimental effects. In *Caenorhabditis elegans*, dual knock down of *decapping protein 1* (*dcp1*) and *decapping protein 2* (*dcp2*) by RNAi can result in partial embryonic lethality (Lall et al., 2005). In *Drosophila*, the early embryonic development is arrested in *dDcp1* mutant backgrounds (Lin et al., 2006). In *Arabidopsis*, cell differentiation is arrested at postembryonic growth in *dcp1* and *dcp2* mutants (Xu et al., 2006). Further, the knockdown of *C. elegans* 5′ to 3′ exoribonuclease *xrn-1* can result in embryos that fail to complete ventral enclosure (Newbury and Woollard, 2004). Therefore, the 5′ to 3′ mRNA degradation pathway plays important roles in various developmental processes among species.

In the 5′ to 3′ mRNA degradation pathway, the 3′ poly(A) tail of the polyadenylated mRNA is usually removed by the deadenylase enzyme complex. After deadenylation, the 5′ cap structure is decapped and the mRNA body is subsequently digested in the 5′ to 3′ direction (reviewed in Parker and Song, 2004). The cleavage of the 5′ cap structure represents a critical step in mRNA degradation and turnover. In yeast, the Dcp1p decapping enzyme is a critical mediator of mRNA decay and an essential component of the 5′ to 3′ mRNA decay pathway (reviewed in Parker and Song, 2004). The two conserved eukaryotic decapping proteins, Dcp1 and Dcp2, function together as a holoenzyme to cleave the 5′ cap structure with Dcp2 being the catalytic subunit. Many of the proteins involved in the 5′ to 3′ mRNA degradation pathways are enriched in cytoplasmic foci termed Processing bodies (P-bodies). Proteins found in P-bodies include the Dcp1 and Dcp2 proteins, stimulators of decapping including Dhh1, Edc3, and Hedls/Ge-1 (also referred to as Edc4), the Lsm1-7 complex, and the 5′ to 3′ exoribonuclease Xrn1 (reviewed in Eulalio et al., 2007a; Fillman and Lykke-Andersen, 2005; Newbury et al., 2006; Parker and Sheth, 2007).

In yeast, evidence suggests that P-bodies can be sites of both mRNA decay and storage because mRNAs have also been shown to re-emerge from P-bodies into polysomes (Brengues et al., 2005). Recently, several additional RNA granules have been described including maternal mRNA granules and neuronal granules which share many fundamental components with P-bodies. For example, the *Drosophila* polar granule, neuronal granule, and P-body share dDcp1 and Me31B, a *Drosophila* homolog of Dhh1p, as common components (Barbee et al.,

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doi:10.1016/j.ydbio.2008.07.033

2006; Lin et al., 2006; Nakamura et al., 2001). Similarly, Staufen, a protein implicated in intracellular RNA transport, is a common component of the *Drosophila* polar granule and neuronal granule (Barbee et al., 2006). The presence of common components among different RNA granules implies that the interchange of components between different RNA granules might be possible.

In *Drosophila* S2 culture cells, decapping proteins together with several effectors of mRNA degradation components are found to be colocalized in cytoplasmic P-bodies (Eulalio et al., 2007b). However, a detailed description of P-bodies in *Drosophila* oogenesis and a biochemical analysis of the decapping proteins are lacking. To characterize the properties of the *Drosophila* P-body in oogenesis, we first showed that dDcp2 contains intrinsic decapping activity by in vitro decapping assays and it colocalizes with dDcp1, Pacman, and Me31B in nurse cell cytoplasm. Similar to previously characterized P-bodies in yeast, *Drosophila* S2 culture cells, and mammalian cells, dDcp1-containing foci (dDcp1 bodies) were sensitive to cycloheximide and RNase A treatment in nurse cell cytoplasm. Together with the fact that the size and number of dDcp1 bodies are affected in *dDcp1*, *dDcp2*, and *pacman* mutant backgrounds, we conclude that dDcp1 bodies in nurse cell cytoplasm represent *Drosophila* P-bodies. In addition, we found that P-bodies are responsive to heat stress and exist in a dynamic state in the cytoplasm of nurse cells. Surprisingly, dDcp1 bodies in the oocytes were distinct from previously characterized P-bodies yet acquire the full complement of P-body components upon deposition of maternal dDcp1 in early stage embryos suggesting that a developmentally regulated conversion occurs between maternal RNA granules and P-bodies during the oogenesis to embryogenesis transition.

Results

dDcp2 contains intrinsic decapping activity

Drosophila melanogaster Dcp1 (dDcp1) is found to be required for the proper degradation of several maternal mRNAs in early embryogenesis (Lin et al., 2006). dDcp1, dDcp2 and Me31B have been shown to colocalize within discrete cytoplasmic foci in *Drosophila* nurse cells (Barbee et al., 2006; Lin et al., 2006), and S2 culture cells (Eulalio et al., 2007b). In addition, these three proteins are present in P-bodies and are involved in mRNA decapping (Coller and Parker, 2004), with Dcp2 being the catalytic subunit (Van Dijk et al., 2002; Wang et al., 2002; Lykke-Anderson, 2002; Steiger et al., 2003). We therefore reasoned that these foci constitute *Drosophila* P-bodies and set out to biochemically determine the decapping properties of dDcp1 and dDcp2.

Four different forms of *Drosophila Dcp2* transcripts at the *dDcp2* locus are reported in FlyBase (FlyBase ID, FBgn0036534 and Fig. S1A). The coding sequences of dDcp2-B and dDcp2-D transcripts are identical. dDcp2-A and dDcp2-C isoforms contain long N-terminal extensions which are unique in Dcp2 variants among species. Through the multiple sequence alignment, dDcp2-B/D is found to be most orthologous to its human and yeast homologs and contains a conserved Nudix hydrolase motif which is required for Dcp2 decapping activity in the yeast and human proteins (Van Dijk et al., 2002; Wang et al., 2002; Lykke-Anderson, 2002; Steiger et al., 2003) (Fig. S1B). To test whether dDcp1 and dDcp2 have intrinsic decapping activity, in vitro decapping assays were performed using His-tagged dDcp1 and His-tagged dDcp2 B-form proteins. In the decapping assays, wild-type His-tagged dDcp1 and double mutated His-tagged dDcp1$^{R57A/G172D}$ contained comparable and background levels of decapping activity indicating that dDcp1 does not contain intrinsic decapping activity (Fig. 1, lanes 2 and 3) under the assay conditions employed. However, the possibility that the His-tag might have a negative effect on dDcp1 decapping activity cannot be excluded. The background activity detected is likely due to copurifying contaminating bacterial protein that can hydrolyze the cap (Liu et al., 2008, in press). In dDcp1$^{R57A/G172D}$ which converts arginine 57 to alanine and glycine 172 to aspartic acid, the two mutated amino acids are conserved among species and disrupt the ability of Dcp1p to stimulate decapping in yeast (Tharun and Parker, 1999). The lack of decapping activity of dDcp1 is consistent with yeast Dcp1p and human Dcp1a which also lack intrinsic decapping activity (Lykke-Anderson, 2002; Van Dijk et al., 2002; Steiger et al., 2003). In contrast to dDcp1, dDcp2 contains robust decapping activity comparable to that observed with human Dcp2 (Fig. 1, lanes 4 and 6). To confirm that the observed decapping activity is a function of dDcp2, two conserved glutamates that are critical for Dcp2 decapping in other organisms (Dunckley and Parker, 1999; Lykke-Andersen, 2002; Van Dijk et al., 2002; Wang et al., 2002) were mutated to glutamines at amino acids 133 and 134 (dDcp2$^{E133/4Q}$). As expected, only background levels of decapping activity, were detected with the dDcp2$^{E133/4Q}$ dual mutation (Fig. 1, lane 5).

Although the yeast Dcp1p protein does not contain intrinsic decapping activity, it is essential for decapping and serves to stimulate Dcp2-mediate decapping enzymatic activity in yeast (She et al. 2004; Steiger et al., 2003). However, a similar stimulatory function has not been observed with human Dcp1 (Lykke-Andersen, 2002; Van Dijk et al., 2002). The discrepancy in the properties of Dcp1 in different species indicates that the mechanism of Dcp2 decapping regulation may be different among species. We therefore tested whether dDcp1 can enhance the decapping activity of dDcp2. Our results indicate that dDcp1 alone cannot enhance dDcp2 decapping activity in an in vitro decapping assay. The decapping activity of recombinant dDcp2 (Fig. 1, lane 9) did not increase upon addition of an increasing titration of His-tagged dDcp1 protein (Fig. 1, lanes 9–12). Collectively, the above data indicate that *dDcp2* encodes a decapping enzyme with intrinsic decapping activity and similar to human Dcp1a, dDcp1 does not detectably stimulate decapping in the absence of other proteins.

Pacman is localized in discrete cytoplasmic foci in nurse cell

P-bodies are sites of mRNA degradation and contain several mRNA degradation factors including the 5′ to 3′ exoribonuclease, Xrn1

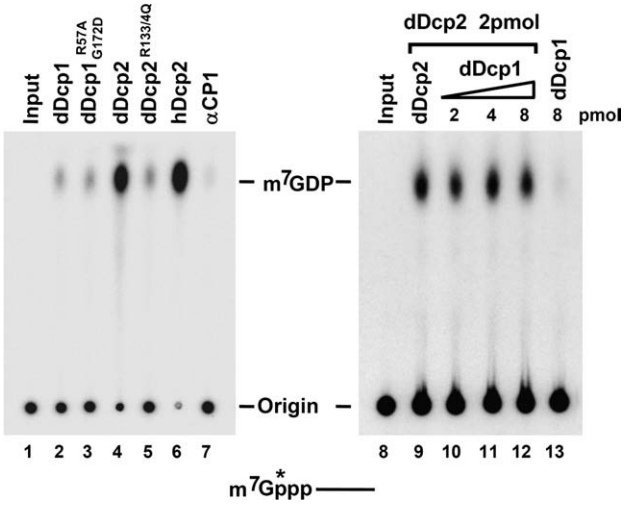

Fig. 1. Recombinant dDcp1 does not contain intrinsic decapping activity and does not stimulate recombinant dDcp2 decapping. Four pmol of His-tagged recombinant wild-type proteins dDcp1, dDcp2, and mutants dDcp1$^{R57A/G172D}$, dDcp2$^{E133/4Q}$ were tested for their ability to hydrolyze cap-labeled RNA in a standard in vitro decapping assay as described in Materials and methods. Recombinant proteins were incubated with ^{32}P-cap-labeled pcP RNA at 37 °C for 30 min. Wild-type dDcp1 and double mutant dDcp1$^{R57A/G172D}$ do not contain decapping activity (lanes 2 and 3). Wild-type dDcp2 carries intrinsic decapping activity (lane 4) as the human decapping enzyme hDcp2 (lane 6). In contrast, double mutant dDcp2$^{E133/4Q}$ lost decapping activity (lane 5). His-tagged αCP1 was used as a negative control (lane 7). Recombinant dDcp2 decapping activity (lane 9) was not increased by adding His-tagged dDcp1 (lanes 10–12). A schematic representation of the ^{32}P-cap-labeled pcP RNA substrate is shown on the bottom where the line represents the RNA body, the m^{7}Gppp denotes the cap and the asterisk indicates the position of the ^{32}P.

(reviewed in Fillman and Lykke-Andersen, 2005). Xrn1 degrades the mRNA body only after the 5′ cap structure is removed (Decker and Parker, 1993; Hsu and Stevens, 1993). Xrn1 is localized to P-bodies (reviewed in Eulalio et al., 2007a; Fillman and Lykke-Andersen, 2005; Parker and Sheth, 2007) and the size and number of P-bodies dramatically increase in an *xrn1* mutant background (Cougot et al., 2004; Eulalio et al., 2007b). This phenomenon can be explained by the accumulation of decapped mRNA and is coincident with the exoribonuclease activity of Xrn1.

Although punctuate Pacman distribution in stage 8 nurse cell cytoplasm has been reported (Barbee et al., 2006), a detailed cytoplasmic distribution of Pacman during oogenesis has not yet been carefully examined. We therefore set out to determine the distribution of the *Drosophila* homolog of Xrn1, Pacman (Chernukhin et al., 2001; Till et al., 1998), during oogenesis. Pacman forms discrete cytoplasmic foci in nurse cells from stage 2 to stage 7 (Figs. 2A–C). At stage 9, Pacman becomes ubiquitously distributed throughout the entire nurse cell cytoplasm and only a few fine dots of Pacman bodies can be observed (Fig. 2D). Both the temporal and spatial distribution of the punctate staining pattern of Pacman in nurse cell cytoplasm is very similar to that of dDcp1 and dDcp2 (Lin et al., 2006). However, unlike dDcp2, Pacman is not localized in nurse cell nuclei (Figs. 2A–D). In the oocyte, Pacman is restricted to the posterior end before stage 7 (Figs. 2A–C). After stage 9, the Pacman staining in the oocyte is very faint and unclear (Fig. 2D). In summary, a developmentally dynamic distribution of Pacman can be observed in different stages (from stage 2 to stage 9) of oogenesis.

As a crucial component of the 5′ to 3′ mRNA degradation pathway, we propose that the punctate Pacman bodies in nurse cell cytoplasm are most likely *Drosophila* P-bodies. However, the possibility that the Pacman bodies in the nurse cell cytoplasm are simply storage sites for Pacman proteins cannot be excluded. Further, the lack of Pacman (Fig. 2D) and dDcp2 (Lin et al., 2006) staining in the oocyte after stage 9 indicates that the posterior localized dDcp1 in the oocyte may not function as foci for mRNA degradation since they lack detectable levels of the two critical nuclease in 5′ to 3′ mRNA decay and could represent a novel form of P-bodies.

dDcp1 is colocalized with dDcp2, Me31B, and Pacman but not Tazman in nurse cell cytoplasm

In yeast and human tissue culture cells, Dcp1 has been considered as a marker for P-bodies (Cougot et al., 2004; Sheth and Parker, 2003;

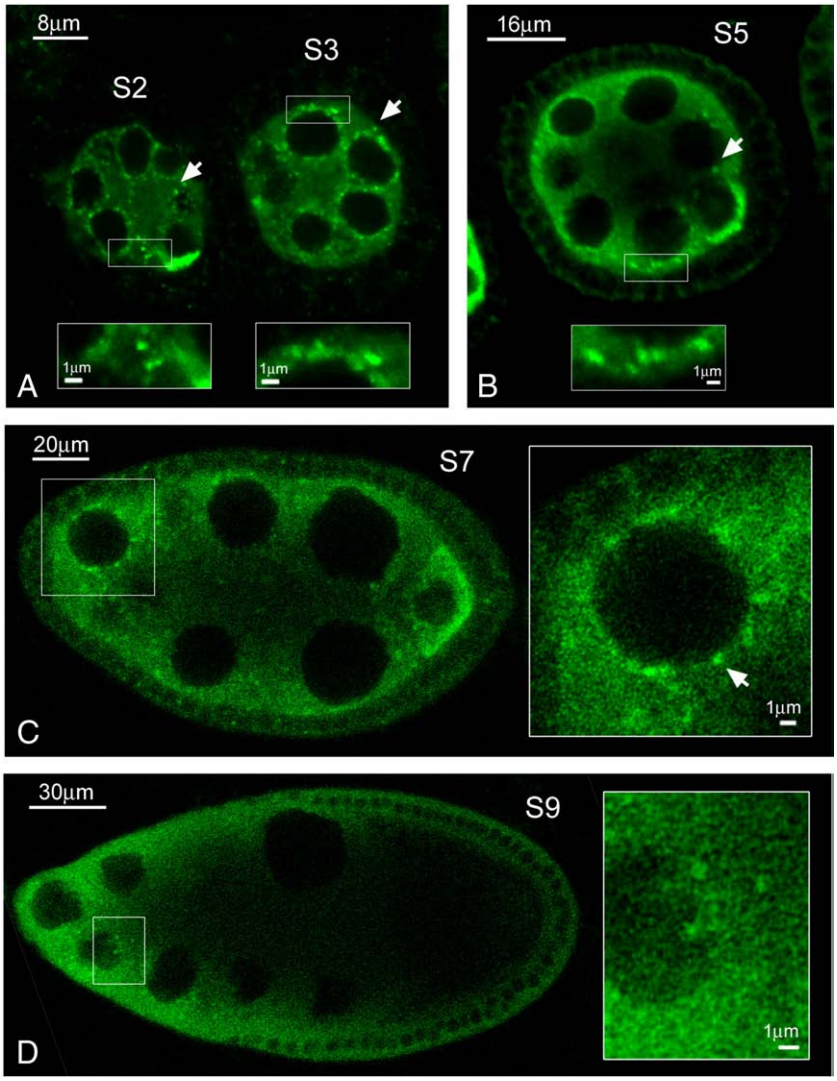

Fig. 2. Pacman localizes within discrete foci in nurse cell cytoplasm. (A–D) Pacman antibody staining in wild-type (A) stage 2 and 3, (B) stage 5, (C) stage 7 and (D) stage 9 egg chambers. (A–C) In nurse cells, Pacman can be detected in punctate particles in the cytoplasm. In the oocyte of early stage egg chambers, Pacman particles are more concentrated in the posterior end. (D) In nurse cells, Pacman signals are greatly reduced in the cytoplasm. In the oocyte, Pacman cannot be clearly detected. In each panel, the indicated inset is reproduced at the bottom (A and B) or at the right (C and D) as enlarged views of the same field. Arrows indicate Pacman foci in the nurse cell cytoplasm.

and reviewed in Eulalio et al., 2007a). As a component of P-bodies, Dcp1 is able to colocalize with Dcp2, Dhh1, Edc3, the Lsm1-7 complex, and the 5′ to 3′ exoribonuclease Xrn1 (reviewed in Eulalio et al., 2007a). In *Drosophila*, dDcp1 colocalizes with both dDcp2 (Lin et al., 2006) and Me31B (Barbee et al., 2006; Lin et al., 2006) in nurse cell cytoplasm and somatic S2 culture cells (Eulalio et al., 2007b). Also, the colocalization between Me31B and Pacman in nurse cell cytoplasm has also been reported (Barbee et al., 2006). To extend the previous studies and further examine whether dDcp1 is an authentic P-body marker in oogenesis, we examined its possible colocalization with other canonical P-body components.

If dDcp1 marks P-bodies which are involved in mRNA degradation, then they would be expected to include the *Drosophila* 5′ to 3′ exoribonuclease, Pacman (Chernukhin et al., 2001; Till et al., 1998). To determine the colocalization between Pacman and dDcp1 in nurse cell cytoplasm, stage 6–7 egg chambers expressing YFP-dDcp1

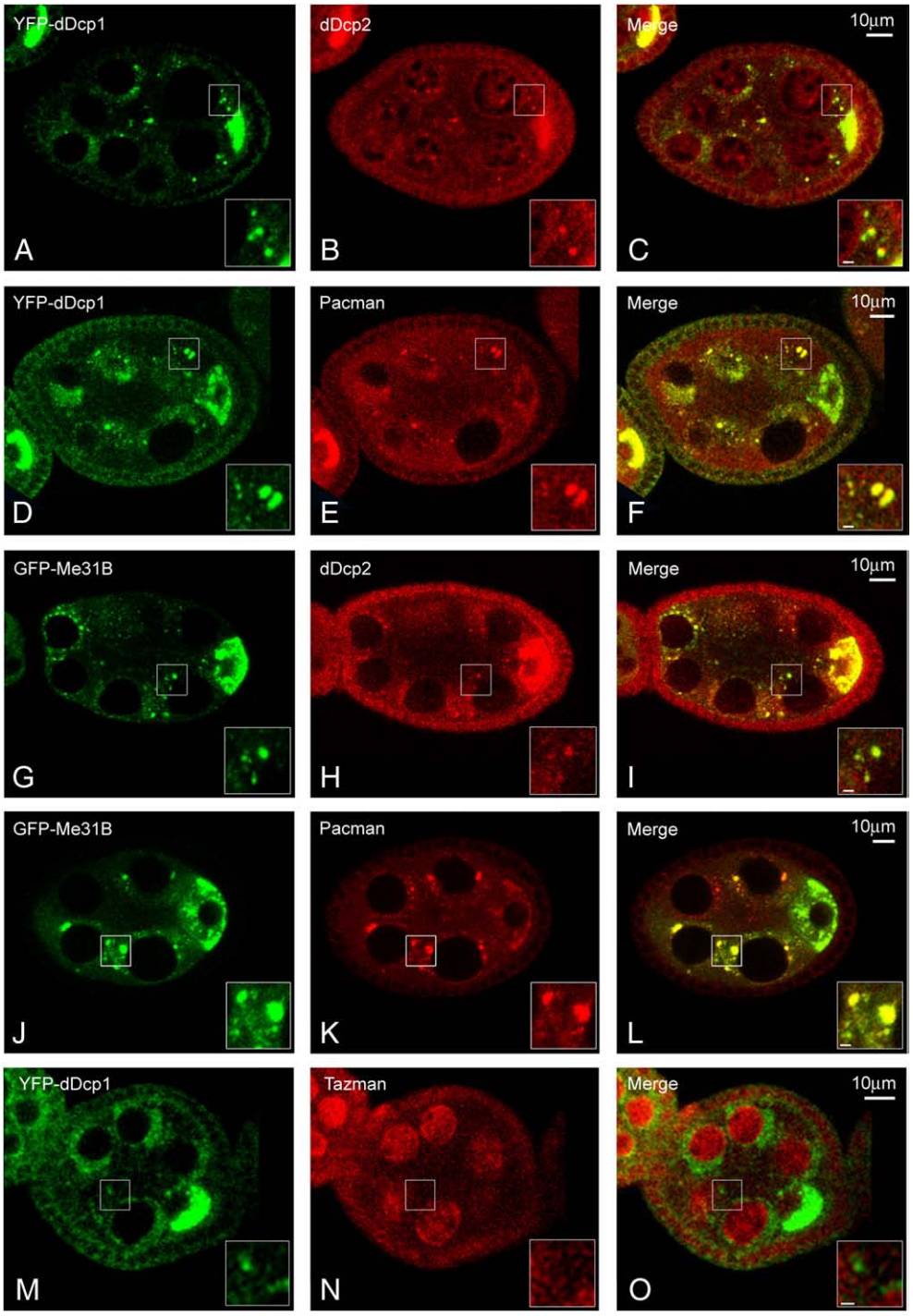

Fig. 3. dDcp1 colocalizes with dDcp2, Pacman, and Me31B but not Tazman in nurse cell cytoplasm. (A–C) YFP-dDcp1 (green) (A) is colocalized with (B) dDcp2 (red) in the stage 7 nurse cell cytoplasm. (C) Merged image. (D–F) YFP-dDcp1 (green) (D) is colocalized with (E) Pacman (red) in the stage 7 nurse cell cytoplasm. (F) Merged image. (G–I) GFP-Me31B (green) (G) is colocalized with (H) dDcp2 (red) in the stage 7 nurse cell cytoplasm. (I) Merged image. (J–L) GFP-Me31B (green) (J) is colocalized with (K) Pacman (red) in the stage 7 nurse cell cytoplasm. (L) Merged image. (M–O) YFP-dDcp1 (green) (M) does not colocalize with (N) Tazman (red) in stage 6 egg chambers. The majority of the Tazman staining signals are localized in nurse cell nucleus. (O) Merged image. In each panel, the indicated inset is reproduced at the lower right as enlarged views of the same field. The scale bar in each inset: 2 μm.

were immunostained with antibodies against either dDcp2 or Pacman. As previously described (Lin et al., 2006), YFP-dDcp1 can colocalize with dDcp2 in the nurse cell cytoplasm (Figs. 3A–C). We also showed that Pacman (Fig. 3E) can colocalize with YFP-dDcp1 (Figs. 3D–F) in nurse cell cytoplasm as well as in the oocyte. To check whether Pacman is also able to colocalize with Me31B, egg chambers expressing GFP-Me31B were examined. The results showed that GFP-Me31B (Fig. 3G) is colocalized with dDcp2 (Fig. 3H). Fig. 3, panels J–L show that Pacman is colocalized with GFP-Me31B (Fig. 3J) in nurse cell cytoplasm as well as the oocyte. Together with the fact that dDcp1 is colocalized with Me31B (Barbee et al., 2006; Lin et al., 2006) and both are colocalized with dDcp2 (Figs. 3A–C and G–I, respectively), we conclude that dDcp1, dDcp2, Me31B, and Pacman are colocalized in particles which resemble P-bodies in the nurse cell cytoplasm. In addition, in stage 7 egg chambers, Pacman is also colocalized with dDcp1 in the ooplasm, albeit at lower levels than dDcp1 and dDcp2.

In yeast, components of the 3′ to 5′ mRNA degradation pathway are absent from P-bodies suggesting that mRNA 3′ end decay does not occur in these foci. For instance, the key exosome subunit Ski7p, which is required for 3′ to 5′ mRNA degradation, does not localize in P-bodies (Sheth and Parker, 2003). To assess whether the *Drosophila* 3′ to 5′ mRNA degradation components are able to localize in P-bodies, we examined the Tazman localization in egg chambers expressing YFP-dDcp1. Tazman is the *Drosophila* homolog of the yeast exosome component Rrp44p which is required for 3′ to 5′ mRNA degradation (Cairrao et al., 2005). In egg chamber expressing YFP-dDcp1, Tazman is mainly localized in the nurse cell nucleus (Fig. 3N) and does not colocalize with dDcp1 (Fig. 3M). Furthermore, only trace amount of Tazman can be observed in the oocyte. This result implies that 3′ to 5′ mRNA degradation most likely does not occur in *Drosophila* P-bodies within nurse cell cytoplasm.

Most dDcp1 bodies do not overlap with Ccr4 in the nurse cells and cellular blastoderm embryo

Exonucleolytic removal of the poly(A) tail is the first step in mRNA decay and appears to be the rate-limiting and regulated step in many cases examined (reviewed in de Moor et al., 2005). In yeast, the major deadenylase complex contains Ccr4p as the catalytic subunit (Chen et al., 2002; Tucker et al., 2001; Tucker et al., 2002). Although the deadenylase complexes have been extensively studied, the location for deadenylation in the cytoplasm is still not very clear. It has been suggested that Ccr4p can transiently localize to the foci containing decapping factors or is present in foci of its own in yeast (Sheth and Parker, 2003). In human cells, hCcr4 is colocalized with cytoplasmic foci enriched in hDcp1a (Cougot et al., 2004). This suggests that the human deadenylation process may also occur in P-bodies.

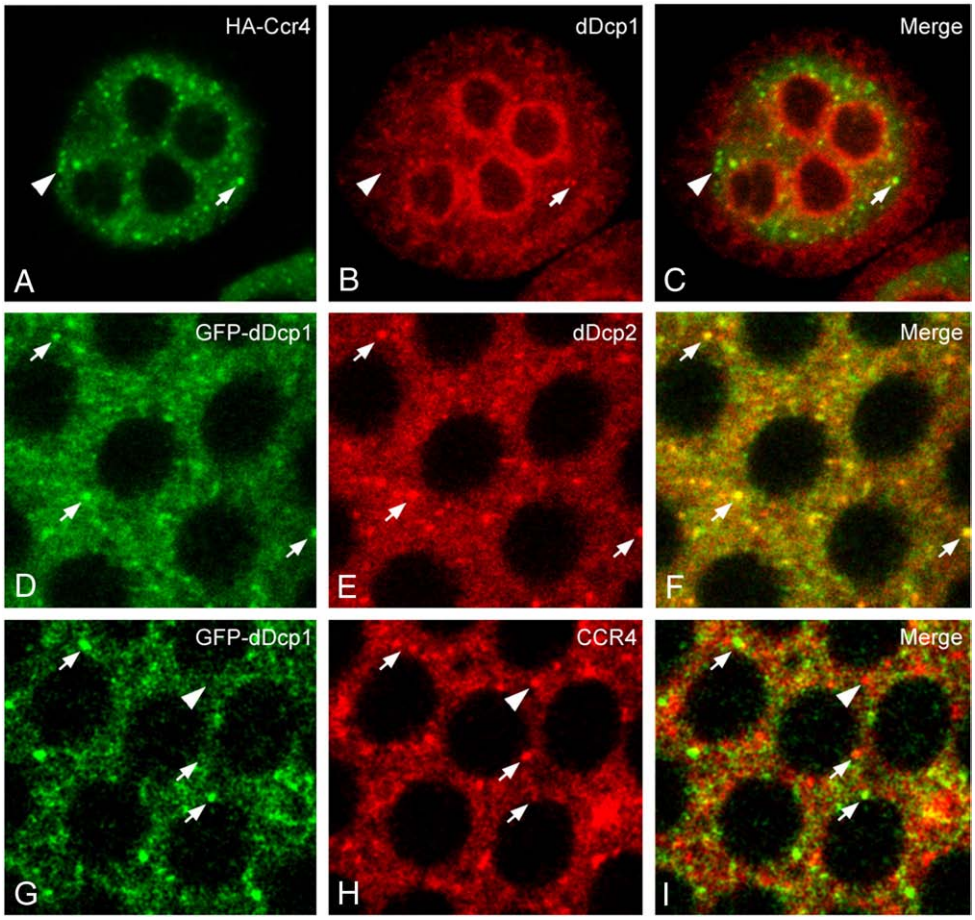

Fig. 4. Most of dDcp1 bodies do not colocalize with Ccr4 in both nurse cells and cellular blastoderm embryos. (A–C) Most of HA-Ccr4 (green) bodies (A) do not colocalize with (B) dDcp1 (red) in the stage 6 nurse cell cytoplasm of the *nanos-Gal4VP16*";;";*UAS-HA-ccr4* egg chambers, while a very limited number of HA-Ccr4 bodies are partially colocalized with dDcp1 (arrow). Note that the oocyte is not included in this optical section. (C) Merged image. Arrowhead indicates distinct HA-Ccr4 body that does not colocalize with dDcp1 body. (D–F) The majority of GFP-dDcp1 bodies (green) (D) are colocalized with (E) dDcp2 bodies (red) in the cellular blastoderm embryos. The embryos were produced by GFP-dDcp1 parents. (F) Merged image. Arrows indicate GFP-dDcp1 bodies that are perfectly colocalized with dDcp2 bodies. (G–I) Most of GFP-dDcp1 bodies (green) (G) are not colocalized with (H) Ccr4 (red) bodies in the cellular blastoderm embryos. The embryos were produced by GFP-dDcp1 parents. (I) Merged image. Arrows indicate that only a few GFP-dDcp1 bodies are partially colocalized with Ccr4 bodies. Arrowhead indicates distinct Ccr4 body that does not colocalize with dDcp1 body.

In *Drosophila*, the deadenylase activity has also been shown to reside with the Ccr4-Not complex which has been found to concentrate in discrete cytoplasmic foci although it is not clear whether these foci are P-bodies (Temme et al., 2004). Moreover, Smaug, a translational repressor, is found to be partially colocalized with Ccr4 and dDcp1 in syncytial blastoderm embryos (Zaessinger et al., 2006). This observation implies the association of Ccr4 and dDcp1 bodies in the cytoplasm of syncytial blastoderm embryo. Since the colocalization between Ccr4 and dDcp1 bodies has not yet been clarified, we stained dDcp1 in HA-Ccr4 expressing egg chambers to exam whether *Drosophila* Ccr4 can colocalize with dDcp1 in nurse cells. In *nanos-Gal4 driven HA-Ccr4* egg chambers, the majority of HA-Ccr4 bodies in nurse cell cytoplasm are not colocalized with dDcp1 (arrowhead in Figs. 4A–C). In contrast to human Dcp1a which is able to colocalize with hCcr4 in culture cells (Cougot et al., 2004), dDcp1 displays a very limited colocalization profile with Ccr4 in the cytoplasm of nurse cells (arrow in Figs. 4A–C; 6 partially overlapped foci out of 48 distinct dDcp1 foci). Therefore we tested whether dDcp1 bodies can colocalize with Ccr4 bodies in embryonic cells. As a control, the localization of dDcp2 bodies in GFP-dDcp1 expressing embryos was examined. In cellular blastoderm embryos, most of the GFP-dDcp1 bodies (arrows in Fig. 4D) are colocalized with dDcp2 bodies

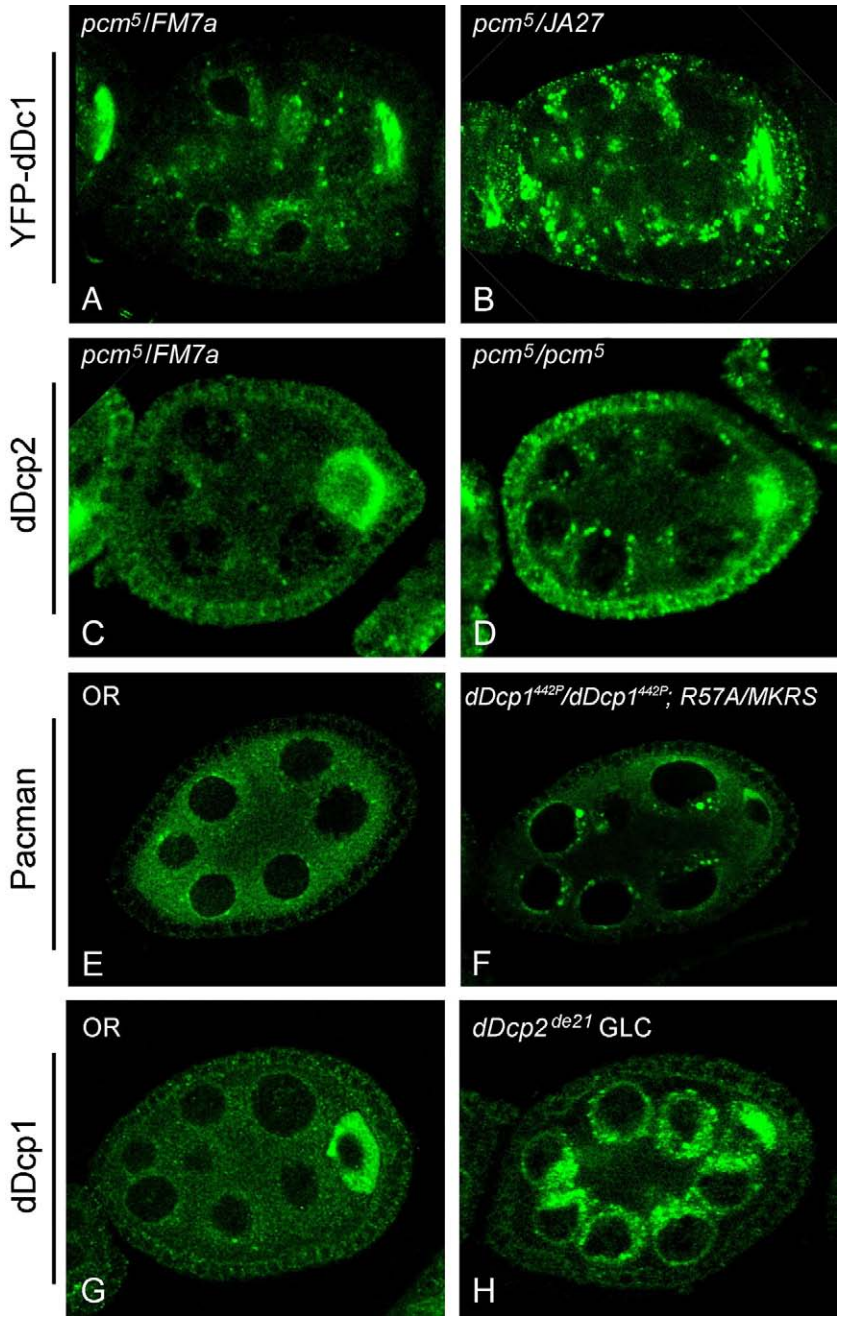

Fig. 5. Depletion of mRNA decay factors affects the size and number of *Drosophila* P-bodies in nurse cells. (A, B) YFP-dDcp1 bodies are increased in *pacman* mutant egg chambers. (A) The direct observation of YFP-dDcp1 in a stage 6, pcm^5/*FM7a*; YFP-dDcp1/CyO egg chamber. pcm^5/*FM7a*; YFP-dDcp1/CyO females were cultivated in 18 °C. (B) The direct observation of YFP-dDcp1 in a stage 6, pcm^5/*JA27*; YFP-dDcp1/CyO egg chamber. pcm^5/*JA27*; YFP-dDcp1/CyO females were cultivated in 18 °C. (C, D) dDcp2 bodies are increased in *pacman* mutant egg chambers. (C) Antibody staining of dDcp2 in a stage 6, pcm^5/*FM7a* egg chamber. pcm^5/*FM7a* females are cultivated in 18 °C. (D) Antibody staining of dDcp2 in a stage 6, pcm^5/pcm^5 egg chambers. pcm^5/pcm^5 females are cultivated in 18 °C. (E, F) Pacman bodies are increased in *dDcp1* mutant egg chambers. (E) Antibody staining of Pacman in a stage 6, wild-type egg chamber. (F) Antibody staining of Pacman in a stage 6, $dDcp1^{442P}/dDcp1^{442P}$; $T2^{R57A}$/*MKRS* egg chamber. (G, H) dDcp1 bodies are increased in *dDcp2* mutant egg chambers. (G) Antibody staining of dDcp1 in a stage 6, wild-type egg chamber. (H) Antibody staining of dDcp1 in a stage 6, $dDcp2^{de21}$ *GLC* egg chamber.

(arrows in Fig. 4E; 12 overlapped foci out of 22 dDcp2 distinct foci). These dDcp1–dDcp2 colocalized foci hence could represent P-bodies in embryonic cells. In contrast, the majority of GFP-dDcp1 bodies (arrowhead in Fig. 4G) do not colocalize with Ccr4 bodies (arrowhead in Fig. 4H). Similar to observations in nurse cells, only a few Ccr4 bodies are intimately associated with dDcp1 bodies (arrows in Fig. 4C; 5 overlapped foci out of 51 distinct Ccr4 foci). Collectively, our data demonstrate that a small subset of foci containing Ccr4 colocalizes with P-bodies while the majority of *Drosophila* Ccr4 cytoplasmic foci are not associated with P-bodies and might be sites of deadenylation or Ccr4 protein storage.

Depletion of mRNA decay factors affects the size and number of P-bodies in nurse cells

The above data suggests that the dDcp1 bodies in the cytoplasm of nurse cells are analogous to P-bodies. Further confirmation of this premise was provided by an alteration of P-body number and size upon disruption of factors involved in mRNA decapping or exoribonuclease activity. Depletion of decapping proteins or the exoribonuclease Xrn1 results in the accumulation of mRNA processing intermediates leading to a dramatic increase in P-body number and size (Sheth and Parker 2003; Cougot et al., 2004). Two *pacman* mutant alleles, the $pacman^3$(pcm^3) and $pacman^5$(pcm^5), were generated by excision of a P-element insertion downstream of the *pacman* gene, between it and the neighboring gene *CG12202*. Western blotting experiments show that pcm^5 homozygous females produce an undetectable amount of full length Pacman protein, whereas pcm^3 mutants express low levels of truncated protein (Fig. S2B). Fig. S2A shows that *pacman* mutant females produce lower number of eggs than isogenic controls, with the fertility of the pcm^5 homozygotes being only 7.1% of that of the isogenic controls. Examination of mutant egg chambers showed that they appeared normal up to stage 8/9 but then rapidly degenerate (Fig. S2C). We have shown that a null mutation in dDcp1 ($dDcp1^{442P}$) caused very low fertility in GLC females and most of the egg chambers are arrested by stage 6 (Lin et al., 2006). Therefore, as for dDcp1, down-regulation of Pacman protein in egg chambers can result in substantial lowering of fertility showing that this exoribonuclease is required for proper oogenesis.

pcm^5 is a cold sensitive *pacman* mutant allele. When pcm^5 is in combination with *JA27*, which is a deletion line that covers the *pacman* locus, and reared in 18 °C, the Pacman protein expression is barely detected (Fig. S2). Therefore, the ovaries from 18 °C reared pcm^5/*FM7a; YFP-dDcp1/CyO* and pcm^5/*JA27; YFP-dDcp1/CyO* females were examined for the YFP-dDcp1 expression. In the nurse cell cytoplasm, the average size and number of YFP-dDcp1 bodies in pcm^5/*JA27* egg chamber (Fig. 5B) is increased to ~180% and ~145% of that in pcm^5/*FM7a* heterozygous control egg chamber (Fig. 5A), respectively. This result indicates that dDcp1 bodies are sensitive to the status of mRNA degradation. Since dDcp2 is able to colocalize with dDcp1 (Figs. 3A–C), we also expect an increase of the size of dDcp2 bodies in *pacman* mutant background. Again, we tested this possibility in pcm^5/*FM7a* and pcm^5/pcm^5 females that reared in 18 °C. As expected, both the average size and number of dDcp2 bodies increased to ~200% in pcm^5 homozygous mutant egg chambers (Fig. 5D) comparing to the pcm^5/*FM7a* heterozygous control (Fig. 5C).

The above data indicate that the size of both dDcp1 and dDcp2 bodies increases in response to a *pacman* mutant. Inversely, Pacman bodies should also increase when the process of mRNA decapping is defective. Our previous studies indicate that $dDcp1^{R57A}$ protein produced in the $T2^{R57A}$ transgene was impaired in its mRNA degradation function (Lin et al., 2006). To create a stock in which the dDcp1 decapping function is impaired, we introduced the $T2^{R57A}$ transgene into *dDcp1* null background ($dDcp1^{442P}$). In $dDcp1^{442P}$/$dDcp1^{442P}$; $T2^{R57A}$/*MKRS* egg chambers (Fig. 5F), Pacman bodies in the nurse cell cytoplasm are increased to ~2.6 times the average size of the wild-type control egg chambers (Fig. 5E). We also examined the dDcp1 distribution in $dDcp2^{de21}$ null background which contains a 6315 bp deletion covering the entire *dDcp2* coding region (Chen, et. al., unpublished). Therefore in the absence of dDcp2 protein, decapping should not occur in the *dDcp2* null mutation and we expect an increase of dDcp1 bodies as a consequence of the accumulation of deadenylated mRNAs. As expected, the average size of dDcp1 bodies is ~6 times increased in $dDcp2^{de21}$ nurse cell cytoplasm (Fig. 5H) compared to wild-type egg control chambers (Fig. 5G). In contrast, the levels of dDcp1 and dDcp2 in the oocyte do not change appreciably, suggesting that these oocyte particles do not behave in the same way as yeast and human P-bodies.

Overall, these results show that the dDcp1/dDcp2/Pacman particles in the nurse cell cytoplasm are dynamic structures and the depletion of mRNA decay factors can increase their size and number, strongly suggesting that they are functionally P-bodies.

YFP-dDcp1 bodies are sensitive to cycloheximide and RNase A treatments

Translational inhibitors such as cycloheximide are known to stabilize mRNAs (reviewed in Jacobson and Peltz, 1996) and inhibit translation elongation by trapping mRNA on polysomes. If structures enriched in mRNA decay factors are actively involved in degradation of mRNAs, we expect their disappearance or reduction after cycloheximide treatment since most of the cellular mRNAs would be trapped within polysomes. In contrast, if they represent the storage sites of mRNA decay factors, we should observe an increase in the size and number after cycloheximide treatment due to the accumulation of unused decay factors. The number and size of P-bodies are reduced after cycloheximide treatment in yeast, *Drosophila* S2, and mammalian cells, suggesting the particles are actively involved in mRNA decay (Cougot et al., 2004; Sheth and Parker, 2003; Eulalio et al., 2007b).

To test whether dDcp1 bodies in ovaries can respond to cycloheximide treatment, hand-dissected ovaries expressing YFP-dDcp1 were treated with 10 μg/ml cycloheximide for 30 min. In the nurse cell cytoplasm of cycloheximide treated egg chambers, we observed a substantial reduction of the average size (60% reduction) and number (40% reduction) of YFP-dDcp1 bodies (Fig. 6B) compared to the DMSO treated control (Fig. 6A). However, the YFP-dDcp1 signals in the oocyte cytoplasm were not reduced significantly (Fig. 6B). To rule out the possibility that the decrease of YFP-dDcp1 bodies was not a consequence of reduced YFP-dDcp1 protein levels, western blot analysis was used to demonstrate that YFP-dDcp1 was stable for at least 1 h following cycloheximide treatment (data not shown). To rule out the possibility that the cycloheximide treatment disrupted the polarity and organization of the oocyte and resulted in a indirect effect on the behavior of YFP-dDcp1 bodies, a posterior localized *oskar* reporter, *osk-(ms2)6* (Supplementary materials and methods) was used to examine the polarity of oocytes after cycloheximide treatment. Our results indicate that the cycloheximide treatment does not affect the posterior localization of the *osk-(ms2)6* reporter in ovaries co-expressing *osk-(ms2)6* and MCP-GFP (Fig. S3). Therefore, the reduction of YFP-dDcp1 bodies after cycloheximide treatments was not caused by an indirect effect. In summary, these results strongly suggest that the dDcp1-containing bodies in nurse cell cytoplasm do not appear to be exclusively protein storage sites for mRNA decay factors. Rather, they are either mRNP storage sites or sites of mRNA decay.

Previous studies in both budding yeast, *Drosophila* S2, and human cells indicate that RNA is required for P-body assembly (Eulalio et al., 2007b; Sen and Blau, 2005; Teixeira et al., 2005). To examine whether RNA is also required for the assembly of the dDcp1 bodies in egg chambers, hand-dissected egg chambers expressing YFP-dDcp1 were treated with RNase A and the YFP emission was directly examined by confocal microscopy. A significant reduction in YFP-dDcp1 bodies was detected in nurse cells with a corresponding increase in diffuse

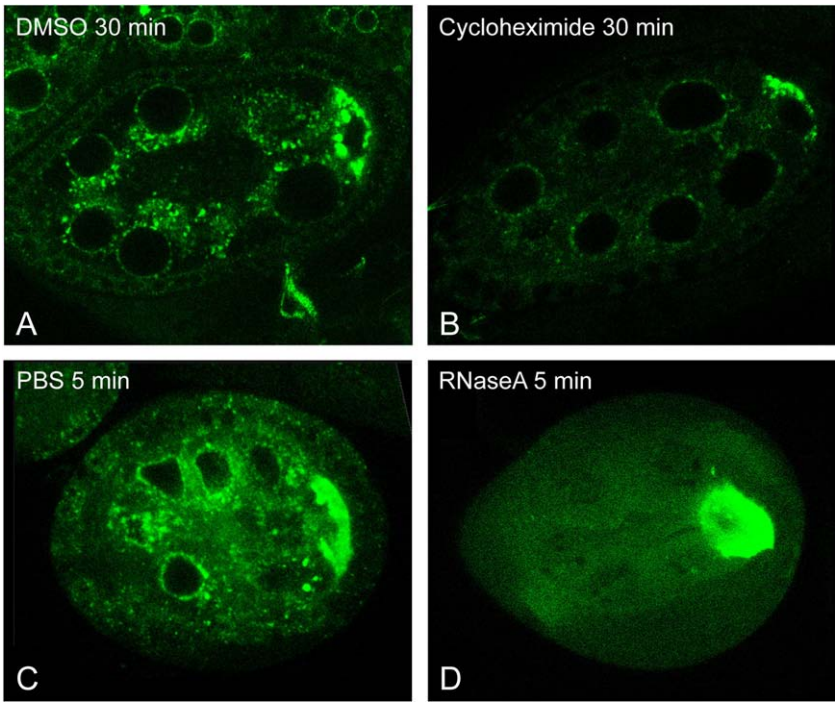

Fig. 6. dDcp1 cytoplasmic foci are sensitive to both cycloheximide and RNase A treatments (A, B) YFP-dDcp1 bodies are greatly reduced after cycloheximide treatment. Hand-dissected YFP-dDcp1 ovaries were treated with either (A) DMSO solvent or (B) 10 μg/ml cycloheximide in DMSO for 30 min. After two washes with PBS, the treated ovaries were directly observed for YFP emission under confocal microscope. (B) The cycloheximide treatment causes the reduction of YFP-dDcp1 particles in nurse cell cytoplasm. Note that the YFP-dDcp1 bodies in the oocyte are not reduced. (C, D) YFP-dDcp1 bodies are not detected following RNase A treatment. Hand-dissected YFP-dDcp1 egg chambers were permeabilized in lysis buffer for 50 s and washed twice with PBS. Egg chambers were then incubated with either PBS only (C) or PBS containing 300 μg/ml RNase A (D) for 5 min. Egg chambers were washed twice in PBS and then directly examined by confocal microscope. The YFP-dDcp1 bodies are no longer detectable and the YFP-dDcp1 proteins are evenly distributed throughout the nurse cell cytoplasm in (D) RNase A treated egg chamber compared with (C) the untreated control egg chamber.

distribution throughout the cytoplasm following RNase treatment (Fig. 6D) while distinctive YFP-dDcp1 bodies were still detected in the PBS treated control (Fig. 6C). These data suggest that the YFP-dDcp1 bodies in nurse cells are RNase sensitive and dependent on RNA for their integrity. In contrast, strong YFP-dDcp1 signal was still detected in the oocyte (Fig. 6D). These data indicate that the dDcp1 bodies in the oocyte are resistant to RNase treatment and their integrity is independent of RNA. They further suggest that dDcp1 bodies in

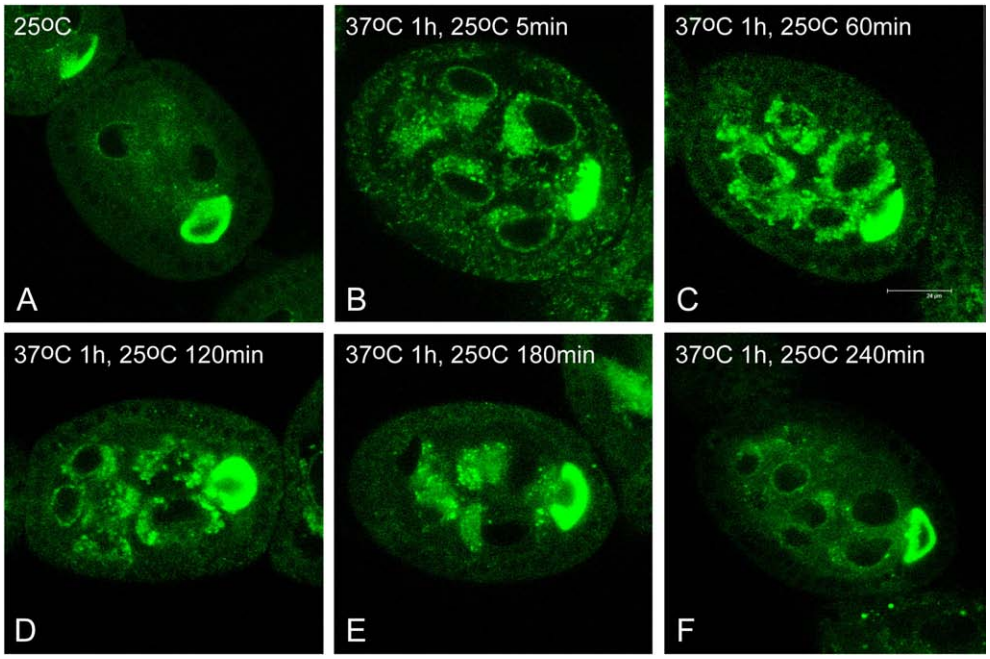

Fig. 7. The amount of YFP-dDcp1 bodies responds to heat-shock treatment. (A) Direct detection of YFP-dDcp1 egg chambers at 25 °C. (B–E) YFP-dDcp1 bodies are dramatically increased in heat-shocked egg chambers. Females expressing YFP-dDcp1 were heat-shocked in 37 °C for 1 h and allowed to recover for (B) 5 min, (C) 60 min, (D) 120 min, and (E) 180 min at 25 °C before dissection. (F) After recovery for 240 min at 25 °C, the amount of YFP-dDcp1 bodies reverted back to the condition similar to that in egg chambers without heat-shock treatment.

oocytes are different from the dDcp1-containing P-bodies in nurse cells. Taken together with the results of cycloheximide and RNase A treatment indicate that the dDcp1 bodies in the nurse cell cytoplasm are *Drosophila* P-bodies with properties similar to yeast, *Drosophila* S2, and human P-bodies.

YFP-dDcp1 bodies are dramatically increased in response to heat stress

P-bodies in budding yeast can respond to several different kinds of stress. For example, the size and number of P-bodies increase in response to glucose deprivation, osmotic stress, and ultraviolet light exposure (Teixeira et al., 2005). Although heat stress has not been reported to lead to an increase in the size or numbers of P-bodies in yeast (Teixeira et al., 2005), the heat stress used was relatively mild (37 °C) so may not have been strong enough to elicit a P-body response (Bond, 2006). In human DU145 cells, exposure to heat-shock (44 °C for 1 h) can result in an increase in the number of P-bodies (Kedersha et al., 2005).

To test the link between heat stress and dDcp1 body number in *Drosophila*, we used YFP-dDcp1 as a marker for *Drosophila* P-bodies in nurse cells to examine the possible response to heat stress. The YFP-dDcp1 females were heat-shocked for 1 h at 37 °C and allowed to recover for varies time points at 25 °C prior to dissection of their ovaries and detection of YFP emission. After 5 min of heat-shock, YFP-dDcp1 foci were dramatically increased and closely associated with nurse cell nuclei (Fig. 7B) compared with the non-heat-shocked control (Fig. 7A). The accumulation of YFP-dDcp1 bodies can still be detected 180 min after heat-shock (Figs. 7B–E). By 240 min following heat-shock, the number and size of YFP-dDcp1 bodies reverted to the status similar to that of untreated control egg chambers (Fig. 7F). The area fraction of aggregated YFP-dDcp1 bodies in nurse cells increased to 8.7% after 5 min of heat-shock (Fig. 7B) from 0.7% of non-heat-shocked control (Fig. 7A). By 240 min following heat-shock, the area fraction of aggregated YFP-dDcp1 bodies in nurse cells is down to 1.6% (Fig. 7F) which is similar to non-heat-shocked control. These results indicate that *Drosophila* P-bodies can respond to heat stress and these effects are reversible (Figs. 7A–F). In contrast, dDcp1 staining in the oocyte did not change appreciably after heat stress. These data confirm that dDcp1 particles in the nurse cell cytoplasm are akin to P-bodies in human cells whereas the accumulation of dDcp1 in the oocyte represents a particle distinct from P-bodies.

dDcp1 colocalizes with GFP-Staufen in the oocyte, while colocalization in the nurse cells is limited

Staufen (Stau) which contains double-stranded RNA-binding domains has been shown to be present in mammalian stress granules, neuronal granules (Thomas et al., 2005), and P-bodies in culture cells (reviewed in Anderson and Kedersha, 2006). In *Drosophila*, Stau is found to reside in neuronal granules related to somatic P-bodies (Barbee et al., 2006) and P-bodies of S2 culture cells (Eulalio et al., 2007b). However, the relationship between Stau and dDcp1 in oogenesis has not been established. Stau is found to be associated with maternal mRNAs including *osk* and responsible for the posterior transportation of *osk* in the oocyte (Ramos et al., 2000; St Johnston et al., 1991). In addition to be a component of P-body, dDcp1 is also a component of *osk* mRNP complex (Lin et al., 2006). Other P-body components, Exu and Me31B which colocalize with dDcp1 in nurse cells (Lin et al., 2006 and Figs. 3J–L, respectively) are also components of an *osk* mRNP complex (Nakamura et al., 2001; Wilhelm et al., 2000). The presence of shared components between the *osk* mRNP complex and P-bodies raises the possibility that certain interactions between these two cytoplasmic RNA granules might exist in nurse cells.

To test whether Stau-containing mRNP complexes are able to interact with dDcp1-containing P-bodies in nurse cells, we examined the possible association and/or colocalization between Stau and dDcp1 in GFP-Stau expressing egg chambers. Large GFP-Stau particles surrounding the nurse cell nuclei together with smaller particles throughout the cytoplasm were observed in nurse cells (Fig. 8A). Through the dual staining of anti-GFP and anti-dDcp1 antibodies, we found that a small subset of GFP-Stau particles in the nurse cell cytoplasm is able to colocalize with dDcp1 particles (arrows in insets of Figs. 8A–C; 9 overlapped foci out of 35 distinct GFP-Stau foci). Although in a small subset, the colocalization between GFP-Stau and dDcp1 raise the possibility that a dynamic transit of shared components from P-body to Stau-containing mRNP in nurse cell cytoplasm might occur. In addition, in the oocyte cytoplasm (arrowhead), GFP-Stau and dDcp1 are completely colocalized. This again implies that the dDcp1 complexes in the oocyte are functionally distinct from those in the nurse cell cytoplasm.

Maternally expressed dDcp1 is able to reform P-bodies with dDcp2 and Pacman in early embryogenesis

Maternal-expressed dDcp1 is necessary for the proper degradation of several maternal mRNAs including *osk*, *bicoid*, and *twine* during maternal-zygotic transition in early embryogenesis since the removal of dDcp1 causes delayed mRNA degradation (Lin et al., 2006). Although dDcp1 is able to colocalize with dDcp2 (Lin et al., 2006 and Figs. 3A–C) and Pacman (Figs. 3D–F) in the stage 2–6 nurse cell cytoplasm, both dDcp2 (Lin et al., 2006) and Pacman (Fig. 2D) are not localized to the posterior pole of the oocyte after stage 9. Furthermore, by using a posterior localized *osk-(ms2)6* reporter, we also demonstrate that dDcp1 is able to colocalize with *osk-(ms2)6* in the posterior

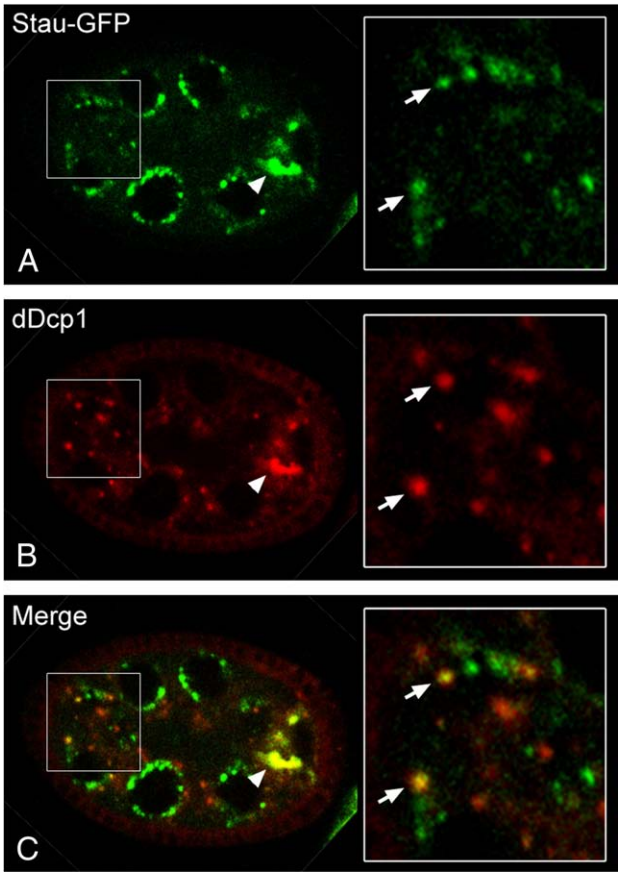

Fig. 8. A small subset of GFP-Stau cytoplasmic particles is able to colocalize with dDcp1 in nurse cells. (A–C) Large GFP-Stau (green) particles surround nurse cell nuclei. Small GFP-Stau particles (arrows) are distributed in the nurse cell cytoplasm. Few (A) GFP-Stau particles in the nurse cell cytoplasm (arrows) are able to colocalize with (B) dDcp1 (red) in the stage 7 egg chamber. (C) Merged image. However, in the oocyte (arrowhead), GFP-Stau and dDcp1 are completely colocalized. In each panel, the indicated inset is reproduced at the lower right as enlarged views of the same field.

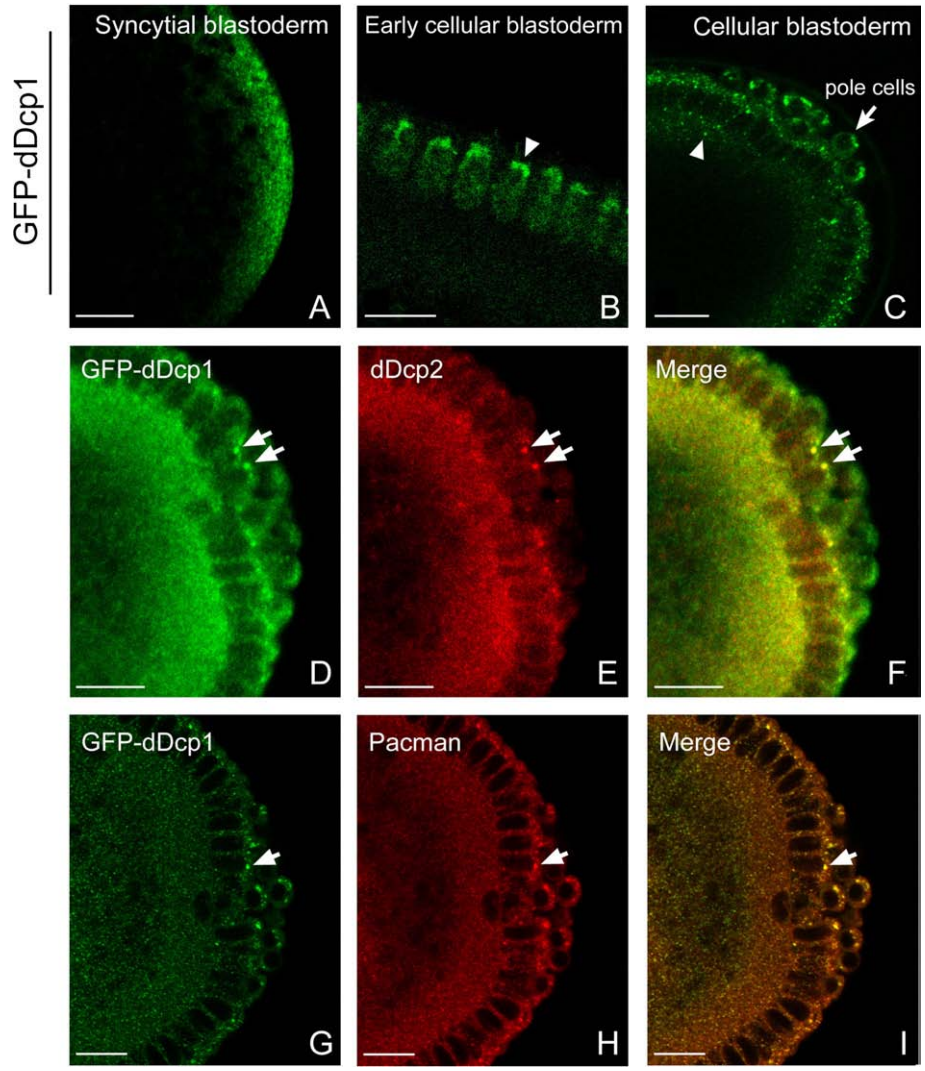

Fig. 9. Maternally expressed GFP-dDcp1 colocalize with dDcp2 and Pacman in early embryogenesis. (A–C) Early stage embryos laid by GFP-dDcp1 females. (A) An early syncytial blastoderm embryo. The maternally expressed GFP-dDcp1 is localized at the posterior end of the embryo. Scale bar: 20 μm. (B) An early cellular blastoderm embryo. Scale bar: 10 μm. (C) A cellular blastoderm embryo. The maternal GFP-dDcp1 (arrowhead) was redistributed in the cytosol of cellularized cells. In addition, GFP-dDcp1 is also localized in the cytoplasm of pole cells (arrow). Green: anti-GFP, scale bar: 20 μm. (D–F) A cellular blastoderm embryo laid by GFP-dDcp1 females. (D) Maternally expressed GFP-dDcp1 is colocalized with (E) dDcp2 in the cytoplasm of cellular blastoderm embryos (arrows). Arrows mark two clear GFP-dDcp1 bodies that are colocalized with dDcp2. (F) Merged images. Green: anti-GFP, red: anti-dDcp2, scale bar: 16 μm. (G–I) A cellular blastoderm embryo laid by GFP-dDcp1 females. (G) Maternally expressed GFP-dDcp1 is colocalized with (H) Pacman in the cytoplasm of cellular blastoderm embryos (arrow). Arrow marks one of the GFP-dDcp1 bodies that colocalized with Pacman. (I) Merged images. Green: anti-GFP, red: anti-Pacman, scale bar: 15.64 μm.

pole of the oocyte after stage 9 while dDcp2 and Pacman do not (Fig. S4). The lack of colocalization among dDcp1, dDcp2, and Pacman in the oocyte, suggested to us that the maternally deposited dDcp1 in the mature egg might be recruited into P-bodies in early embryogenesis for maternal mRNA degradation. To evaluate this possibility, embryos obtained from females carrying a maternal-specific GFP-dDcp1 transgene were examined. The maternal *α4 tubulin* promoter driven GFP-dDcp1 ensures that GFP-dDcp1 observed in early embryos is derived from the oocyte but not from the zygotic *dDcp1* expression. In early syncytial blastoderm embryo, GFP-dDcp1 is localized at the apical region of the embryo (Fig. 9A). During cellularization, GFP-dDcp1 is concentrated at the apical pole of the elongated nuclei (Fig. 9B) which corresponds to the position of the microtubule organization center (Callaini and Anselmi, 1988; Kellogg et al., 1991). In the cellular blastoderm embryo, GFP-dDcp1 is redistributed and forms discrete cytoplasmic foci (Fig. 9C) throughout the cellularized cells. Importantly, the timing of the formation of these discrete GFP-dDcp1 cytoplasmic foci is coincident with that for maternal mRNAs degradation including *osk*, *bicoid*, and *twine* (Lin et al., 2006). We therefore speculate that the maternal GFP-dDcp1 bodies observed in cellular blastoderm embryos are P-bodies. To further substantiate this possibility, embryos expressing maternal GFP-dDcp1 were dual stained with either anti-GFP and anti-dDcp2 antibodies or anti-GFP and anti-Pacman antibodies. As expected, the maternal GFP-dDcp1 bodies in the cellular blastoderm embryos can colocalize with dDcp2 (Figs. 9D–F) and Pacman (Figs. 9G–I). Collectively with our previous observations (Lin et al., 2006) and the correlation of the timing of P-body re-formation and maternal mRNA degradation, we propose that the maternally produced dDcp1 in the egg might be recruited into P-bodies and contribute to the degradation of a certain subset of maternal mRNAs in early embryogenesis.

Discussion

dDcp2 intrinsic decapping activity and its relationship with dDcp1

To gain a clearer understanding of the developmental expression and function of the dDcp1 and dDcp2 decapping proteins, we tested their

ability to hydrolyze an mRNA cap and also determined their localization throughout *Drosophila* oogenesis and early embryogenesis.

We first demonstrated that the *Drosophila* Dcp2 protein possesses intrinsic decapping activity using in vitro decapping assays (Fig. 1). Consistent with both yeast and human Dcp1 proteins, intrinsic decapping activity was not detected for dDcp1 (Fig. 1). Previous studies from yeast indicate that Dcp1p can enhance the decapping activity of Dcp2p (Beelman et al., 1996; Steiger et al., 2003) possibly by promoting or stabilizing a catalytically active conformation of Dcp2p (She et al. 2008). However, we did not detect a stimulation of dDcp2 decapping at least in vitro (Fig. 1) despite their colocalization in the nurse cell cytoplasm (Figs. 3A–C). One possibility for the lack of stimulation could be that additional proteins might be required for a stable interaction between dDcp1 and dDcp2. Human Dcp2 decapping is stimulated by the Hedls/Ge-1 protein (Fenger-Gron et al., 2005; Yu et al., 2005). Hedls/Ge-1, which contains an N-terminal WD40 motif and C-terminal domains characterized by a repeating $\psi(C_{2-3})$ motif, promotes the association between Dcp1a and hDcp2 and is proposed to facilitate hDcp2 decapping (Fenger-Gron et al., 2005; Yu et al., 2005). A likely possibility remains that the *Drosophila* homolog of Hedls/Ge-1 (*CG6181*) or a functionally equivalent protein, that was absent in the in vitro assays, might be necessary to facilitate an interaction between dDcp2 and dDcp1 and thereby stimulate dDcp2 decapping.

P-bodies might be sites of mRNP assembly

Consistent with dDcp1 bodies in S2 culture cells (Eulalio et al., 2007b), our data presented here indicate that dDcp1 bodies in the nurse cell cytoplasm can represent P-bodies in oogenesis. Studies indicate that activators of mRNA decapping such as yeast Dhh1p and mammalian RCK/p54 have been found to be translational repressors (Coller and Parker, 2005). These findings led to a model in which the translational status of a cytoplasmic mRNA is the consequence of competition between the translational apparatus and the repression apparatus (Coller and Parker, 2005). Furthermore, a reciprocal movement of mRNAs between polysomes and P-bodies is possible (Brengues et al., 2005). Therefore, a dynamic switch of mRNAs among translational activation, translational repression, and degradation is conceivable. In this study, we demonstrated that dDcp1 bodies can partially colocalize with GFP-Stau in nurse cell cytoplasm (Fig. 8). Together with the fact that Stau is a double-stranded RNA-binding protein (Ramos et al., 2000) and a common component of RNA granules including stress granule, neuronal granule and somatic P-body in mammalian and *Drosophila* S2 cells (Anderson and Kedersha, 2006; Barbee et al., 2006; Eulalio et al., 2007b; Kedersha et al., 2005), it is possible that P-bodies in the nurse cell cytoplasm could be sites of Stau-containing maternal mRNP assembly.

dDcp1 bodies in the oocyte appear to be modified P-bodies

In the oocyte, accumulation of Pacman at the posterior pole is especially obvious in stage 2–6 egg chambers (Figs. 2A, B and3K). In addition, strong dDcp1, dDcp2, and Me31B signals can be detected at the posterior pole of the oocyte in similar stages (Fig. 3). Considering the inactive biological nature and maternal mRNA deposition and storage functions of oocyte, the requirement for the mRNA degradation in the oocyte is assumed to be very limited. If this is the case, the accumulated dDcp1, dDcp2, Me31B, and Pacman in the oocyte before stage 6 may not function as foci for mRNA degradation. One possibility is that Me31B which colocalize with dDcp1 bodies in stage 2–6 oocytes (Lin et al., 2006 and Figs. 3A–L) contributes to formation of a translationally repressed complex. In *Drosophila*, Me31B is involved in translational repression of maternal mRNAs (Nakamura et al., 2001) through the interaction with eIF4E, Cup, and Bruno (Nakamura et al., 2004; Wilhelm et al., 2003). In addition, Me31B can participate with an FMRP-associated translational repression function in developing eye imaginal discs and in bantam miRNA-mediated translational repression in wing imaginal discs (Barbee et al., 2006). Therefore, it is possible that the posterior accumulated mRNA decay factors in stage 2–6 oocyte may represent translational repressed complexes and function to store maternal mRNAs. This hypothesis is further supported by the fact that yeast P-bodies can function as storage sites for translationally repressed mRNAs (Brengues et al., 2005). Our observations that the YFP-dDcp1 signals in stage 6 oocyte are insensitive to cycloheximide (Fig. 6B), RNase A treatment (Fig. 6D) and heat-shock (Fig. 7) also support this hypothesis. Following stage 9, dDcp1 is localized to the posterior end of the oocyte (Lin et al., 2006 and Figs. S4A–C) and colocalized with Stau (Lin et al., 2006). In contrast, both dDcp2 (Lin et al., 2006 and Figs. S4D–F) and Pacman (Fig. 2D and Figs. S4G–I) are not localized to the posterior pole of the oocyte after stage 9. This argues that the posterior localized dDcp1 bodies in the oocyte most likely do not function as foci for mRNA degradation since they lack the 5′ to 3′ mRNA degradation enzymes, dDcp2 and Pacman.

In conclusion, our data raises the intriguing possibility that dDcp1 may function in translational repression and/or on maternal RNA granule storage in stage 2–6 oocyte. It also suggests that, in *Drosophila* oocyte after stage 9, dDcp1 is not an appropriate marker to represent canonical P-bodies involved in mRNA degradation. Further, the posterior localized dDcp1 bodies in the oocyte could be considered as a novel form of modified P-bodies ready for its conversion into zygotic P-bodies in early embryogenesis (see below).

The possible developmental conversion between dDcp1-containing maternal RNA granule and zygotic P-body

In stage 9–10 oocyte, dDcp1 does not colocalize with dDcp2 and Pacman (Lin et al., 2006; and Fig. 2) while they reunite again in early embryogenesis (Fig. 9). These results suggest that there is a dDcp1 independent function of dDcp2 and Pacman in oogenesis and a sophisticated regulation of mRNA degradation during *Drosophila* oogenesis. The timing of the re-formation of P-bodies in the early embryo coincides with the degradation of maternal mRNAs during maternal-zygotic transition. Further, the degradation of *bicoid*, *osk*, and *twine* mRNAs during maternal-zygotic transition in early embryogenesis is dependent on dDcp1 activity (Lin et al., 2006). Considering dDcp1 is a component of *osk* mRNP, we suspect that a direct conversion from the dDcp1-containing maternal RNA granules to the zygotic P-body in early embryogenesis may be responsible for the efficient and dramatic degradation of maternal mRNAs at 2–3 h AEL (stage 14) (Lin et al., 2006). This proposal is supported by the fact that shared mRNA decay components in addition to dDcp1 are found in both maternal RNA granules and P-bodies. For example, Me31B is a component of the *osk* mRNP complex in the oocyte (Nakamura et al., 2001) and its counterpart, Dhh1p/Rck, resides in yeast and human P-bodies (Sheth and Parker, 2003; Cougot et al., 2004).

Collectively, our data indicate a fluctuation of P-body number and size from oogenesis to embryogenesis. In the nurse cell cytoplasm, the colocalization among dDcp1 and other mRNA decay components including dDcp2 and Pacman in the stage 6–7 nurse cells (Fig. 3) implies the presence of P-bodies with mRNA degradation function, although their mRNA storage function cannot be excluded. In the oocyte after stage 9, dDcp1 (Figs. S4A–C and Lin et al., 2006) and Me31B (Nakamura et al., 2001) are able to localize to the posterior pole of oocyte. In contrast, dDcp2 and Pacman which contain the major degradation enzyme activities do not localize to the posterior pole of the stage 9 oocyte (Figs. S4D–I and 2D and Lin et al., 2006). This suggests that accumulation of dDcp1 in the oocyte at late stages is in a developmental state without mRNA degradation function. In early embryogenesis, we observed the colocalization of maternally expressed dDcp1 with dDcp2 (Figs. 9D–F), and Pacman (Figs. 9G–I). This re-formation of maternally produced dDcp1 with dDcp2 and Pacman indicates the re-formation of P-bodies in early embryogenesis, which might be responsible for the abrupt degradation of certain maternal mRNAs at the cellularization stage mediated by dDcp1 (Lin et al., 2006). This dynamic change and

conversion of P-bodies illustrate the requirement of a tight regulation of mRNA turnover during development.

Drosophila P-bodies can response to heat stress

In Fig. 7, we show that heat stress induces a clump of P-body aggregation around nurse cell nuclei. Moreover, this aggregation can be reversed (Figs. 7A–F). Morphologically, this dynamic nature resembles the behavior of mammalian stress granules that function to store translationally stalled housekeeping mRNAs in stress conditions (reviewed in Anderson and Kedersha, 2002; Kedersha and Anderson, 2002). We therefore suspect the aggregation of P-bodies around nurse cell nuclei under heat stress might be functionally related to mammalian stress granules in certain way. One possibility is that these *Drosophila* P-bodies in the nurse cell cytoplasm can be a functional homolog of mammalian stress granules. Another possibility is that distinct stress granules co-exist with P-bodies and cooperate with P-bodies in response to stress conditions. Till now, the functional homologs of mammalian stress granules have not yet been identified in *Drosophila*. To distinguish these two alternatives, it is critical to identify the existence of *Drosophila* homologs of mammalian stress granule components.

Materials and methods

Drosophila stocks

Oregon R (OR) is used as the wild-type strain. Fly stocks were raised at 25 °C on standard cornmeal medium. The following transgenic stocks were used in this study: P{w+; *GFP-Me31B*} (Nakamura et al., 2001), P{w+; *UASp-HA-ccr4*} (Semotok et al., 2005), P{w+; *YFP-dDcp1*} (Lin et al., 2006), P{w+; *mat-tub-alpha4:GFP-dDcp1*} (this line contains a *GFP-dDcp1* transgene driven from the maternal *α4 tubulin* promoter), P{w+; *mat-tub-alpha4:GFP-Staufen*} (Schuldt et al., 1998), and *nanos-Gal4VP16* (Van Doren et al., 1998). For *pacman* mutants, stock 11456 (obtained from Bloomington *Drosophila* Stock Center) carrying the P insertion *P{EP}EP1526* was used to create pcm^3 *and* $pcm^5$by imprecise P-element excision using standard protocols. These mutants were further characterized at the molecular level by amplification of DNA at either side of the breakpoint using primers jes1 5′-TCCCGATCACGATGAAGACC-3′ and big1 5′-ACTGCCGCCTCAGATCTG-3′. Full details on the generation of *pacman* mutant alleles are given elsewhere (Grima et al., 2008).

Fertility experiments

Flies homozygous for pcm^5 or pcm^3 were mated to wild-type males (OregonR) and number of eggs counted over 10 h at 25 °C. Egg chambers were visualised by dissecting females in Ringers solution fixing egg chambers (Verheyen, E. and Cooley, L. (1994) "Looking at oogenesis" Methods Cell Biol. vol 44 545–561.), staining with DAPI (1 μg/ml for 5 min) and mounting in 70% glycerol.

In vitro decapping assays

In vitro decapping assays were carried out using ^{32}P-cap-labeled pcP RNA with the indicated protein as previously described (Wang et al., 2002). The decapping products were resolved by polyethyleneimine (PEI) cellulose thin-layer chromatography (TLC) developed in 0.75 M LiCl. TLC plates were exposed to PhosphorImager. The full length coding sequences of HA-tagged dDcp1 and dDcp2 were subcloned form EST clone GH04763 or EST clone SD14939 respectively into the pRest vectors. The mutant plasmids pRSET-dDcp1$^{R57A/G172D}$ and pRSET-dDcp2$^{E133/4Q}$ were generated by site-directed mutagenesis using QuikChange multi site-directed mutagenesis kit (Strategene) according to the manufacturer's manual. All plasmids were confirmed by sequencing. His-tagged proteins were expressed in *Escherichia coli* and purified with His Bind Resin according to the manufacturer's manual (Novagen; San Diego, CA).

Immunofluorescence staining and image analysis

Ovaries from 1- to 3-day old females were dissected in PBS on ice and fixed for 20 min in fixative (600 μl of heptane, 200 μl of 2% paraformaldehyde in PBS, and 1 μl of NP-40). After 3 washes with PBT (PBS plus 0.2% Tween 20), the fixed ovaries were incubated in PBT containing 1% Triton X-100 for 1 h. Ovaries were then blocked for 3 to 5 h in 5% normal goat serum in PBT, and incubated overnight at 4 °C in primary antibody diluted in PBT (1:200 for rabbit anti-Ccr4 antibody (Temme et al., 2004); 1:20 for rabbit anti-dDcp1 antibody; 1:100 for rabbit anti-dDcp2 antibody; 1:500 for rabbit anti-Pacman antibody; 1:50 for rabbit anti-Tazman antibody (Cairrao et al., 2005); 1:100 for chicken anti-GFP antibody; 1:100 for rat anti-HA antibody). The ovaries were then washed 3 times for 20 min each in PBT, and then incubated for 2 h at room temperature in secondary antibody in PBT. Following three 30 min washes in PBT, the ovaries were mounted in anti-fade mounting solution (PBS containing 50% glycerol and 2% DABCO). For preparation of an antibody to Pacman, a cDNA encoding a 54 kDa C-terminal portion of Pacman was expressed as a His-tag fusion protein in *E. coli* using the expression vector pET28a. The histidine tag was removed by thrombin treatment and the *pacman* protein fragment cut from the gel for use in raising antibodies. The antibody was prepared by the company Eurogentec.

The image analysis was performed by using the software ImageJ which is a public domain Java image processing program developed by Wayne Rasband (http://rsb.info.nih.gov/ij/index.html). The distinct particles were analyzed by using the "Analyze particles" command of ImageJ. The colocalization of cytoplasmic foci was analyzed by the "Colocalization" plug-in that developed by Pierre Bourdoncle.

RNase A and cycloheximide treatment of dissected egg chambers

For RNase A treatment, hand-dissected egg chambers that are expressing EYFP-dDcp1 were permeabilized in lysis buffer (100 mM potassium phosphate, pH 7.8, with 0.2% Triton X-100) for 50 s and washed twice with PBS. Egg chambers were then incubated with either PBS or PBS containing RNase A (300 μg/ml) for 5 min. Egg chambers were washed twice in PBS and then directly examined by confocal microscopy. For cycloheximide treatment, hand-dissected egg chambers expressing EYFP-dDcp1 were incubated in Schneider's *Drosophila* medium (GIBCO) containing 10 μg/ml of cycloheximide for 30 min. Egg chambers were then washed with PBS twice and directly examined by confocal microscope.

Heat-shock of YFP-dDcp1 flies

We incubated well-fed female flies expressing EYFP-dDcp1 in 37 °C for 1 h. After heat-shock, female flies were recovered in 25 °C for 15 min. The ovaries were then dissected for direct observation of EYFP-dDcp1 by confocal microscopy.

Acknowledgments

We thank Chao-Han Chen who provided the unpublished $dDcp1^{de21}$ mutant allele. We are grateful to Akira Nakamura, Anne Ephrussi, Daniel St Johnston, Elizabeth Gavis, Elmar Wahle, Howard D. Lipshitz, Jennifer L. Semotok, Robert H. Singer, and the Bloomington stock center for their generous gifts of fly stocks and antibodies. We thank Dr. France Docquier for advice on the P-element excision work and Professor Roy Parker for advice and encouragement. We are also grateful to Dawn Field, Helen Glenwright, Cathy Browne, Alex Blundell and Sarah Allan for technical help. We thank the Confocal Microscope Lab, Instrumentation Center, NTU, for assistance with microscopy. This

project is supported by the National Sciences Council (NSC93-2311-B-002-027; NSC94-2311-B-002-032), Taiwan, ROC to Tze-Bin Chou; National Taiwan University (postdoctoral fellowship 95R0044), Taiwan, ROC to Ming-Der Lin; National Institutes of Health grant GM67005 to Megerditch Kiledjian; the UK Biotechnology and Biological Sciences Research Council to Sarah F. Newbury.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2008.07.033.

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