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***Drosophila* patterning is established by differential association of mRNAs with P bodies**

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Abstract

The primary embryonic axes in flies, frogs and fish are formed through translational regulation of localized transcripts before fertilization¹. In *Drosophila*, the axes are established through the transport and translational regulation of *gurken* (*grk*) and *bicoid* (*bcd*) messenger RNA (mRNA) in the oocyte and embryo¹. *bcd* and *grk* mRNA are both translationally silent while being localized within the oocyte along microtubules by cytoplasmic Dynein¹⁻⁴. Once localized, *grk* is translated at the dorsoanterior of the oocyte to send a TGF- α signal to the overlying somatic cells⁵. In contrast, *bcd* is translationally repressed in the oocyte until its activation in early embryos to form an anteroposterior morphogenetic gradient⁶. How this differential translational regulation is achieved is not fully understood. Here, we address this question using ultrastructural analysis, super-resolution microscopy and live cell imaging. We show that *grk* and *bcd* ribonucleoprotein (RNP) complexes associate with electron dense bodies that lack ribosomes and contain translational repressors, characteristic of Processing bodies (P bodies), which are regions of cytoplasm where translational decisions are made. Endogenous *grk* mRNA forms dynamic RNP particles that become docked and translated at the periphery of P bodies, where we show that the translational activator Orb/CEPB and the anchoring factor Squid (Sqd) are also enriched. In contrast, an excess of *grk* mRNA becomes localized inside the P bodies, where endogenous *bcd* mRNA is localized and translationally repressed. Interestingly, *bcd* mRNA dissociates from P bodies in embryos following egg activation, when it is known to become translationally active. We propose a general principle of translational regulation during axis specification involving remodeling of transport RNPs and dynamic partitioning of different transcripts between the translationally active edge of P bodies and their silent core.

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At the dorsoanterior corner of the oocyte, localized *grk* mRNA is associated with electron dense structures⁷, which were originally characterized in nurse cells⁸ and later in the oocyte as sponge bodies⁷. These structures have subsequently been shown to share at least some components with yeast and mammalian P bodies⁹⁻¹¹. We, therefore, refer to sponge bodies as P bodies. To test whether, like *grk*, *bcd* mRNA is associated with P bodies, we used *in situ* hybridization with antisense *bcd* probe combined with immuno-electron microscopy on frozen sections (ISH-IEM)¹². We found that *bcd* mRNA is also associated with P bodies in mid-oogenesis (Fig. 1a and Supplemental Fig. 1). Closer examination revealed that *grk* mRNA is present along the periphery of the P body (Fig. 1b,c), whereas *bcd* is mostly present within its interior (Fig. 1a,b). To characterize this further, we covisualized endogenous *grk* mRNA and a DEAD-Box helicase Maternal expression at 31B (Me31B), a well established P body marker¹³. We found that *grk* mRNA appears to localize just outside a 'core' zone of concentrated Me31B labeling (Fig. 1d). We then performed a quantitative analysis of the density of gold as a function of the distance from the border of the P body. This analysis applied to Me31B and Orb (Supplemental Fig. 2b,b', Supplemental methods), shows that P bodies are organized into two different regions: a 'core' containing the majority of Me31B and 'edge', the outermost 60-80nm of electron dense material, defined as 70nm for convenience of analysis (Fig. 1e, Supplementary Fig. 2c). We found that, relative to the surrounding cytoplasm, *grk* mRNA is 23 times more concentrated at the edge of the P body whereas *bcd* is 6 fold more concentrated in the P body core (Fig. 1e).

While ISH-IEM provides excellent ultrastructural preservation and resolution, it only represents a two dimensional view and has limited sensitivity of detection of RNA, since gold conjugates only penetrate the first 5nm of the ultrathin 60nm frozen section¹². We, therefore, used the complementary method of structured illumination microscopy (3D-SIM), which provides double the conventional resolution of light microscopy in each dimension¹⁴. To covisualize P bodies and mRNA, we detected P bodies with a GFP fusion to the DEAD-Box helicase Maternal expression at 31B (Me31B::GFP)¹³ at the same time as tagging *grk* mRNA with 12 copies of the MS2 binding loop or *bcd* mRNA with 6 copies of MS2, both encoding functional genes^{2,15,16}. The tagged mRNA was decorated with MS2-coat protein (MCP) fused to fluorescent proteins^{2,15-17}, subsequently referred to as *grk*GFP*, *RFP* or *mCherry*). We found that *grk*mCherry* is excluded from the core of the P bodies and interdigitates with Me31B::GFP, with a slight overlap at the interface, which we interpret as consistent with the ISH-IEM results (Fig. 1f). In contrast, *bcd*RFP* overlaps significantly with the P body core in both techniques (Fig. 1g).

We next tested whether the differences we observe in the distribution of *grk* and *bcd* mRNAs relative to P bodies can be explained by their distinct transport and anchoring dynamics. We performed rapid single and multicolor wide-field fluorescence microscopy in living stage 8/9 oocytes (Fig. 2a-e, Supplementary Movie 1-6). We observed three distinct populations of mRNA particles: 'active transport particles' moving along linear paths characteristic of transport by molecular motors; 'paused transport particles' showing a complex constrained motion; and 'docked *grk*' statically associated with P bodies (Supplemental Fig. 3a,b). Active transport particles of *grk* mRNA and *bcd* mRNA do not colocalize with Me31B, but rather move in the space between P bodies with an average

velocity of 0.5 $\mu\text{m/s}$ (Fig. 2c,e,f, Supplementary Movie 4,6). 41% of *grk* particles dock at the edge of P bodies (Fig. 2d, Supplementary Movie 5). In contrast, out of 155 *bcd* transport particles, none showed *grk*-like docking at the edge of the P bodies in stage 8/9 oocytes. A quantitative analysis of particle motility, by mean squared displacement confirmed the difference between motile and docked particles (Supplemental materials and methods). We suggest that *bcd* mRNA rapidly integrates into the P body core upon contact with the P bodies, whereas *grk* mRNA particles remain docked on their outside.

Our covisualization of mRNA and Me31B showed that mRNA particles move independently of Me31B. To determine whether Me31B is also dynamic and to establish how its distribution in P bodies is maintained, we first visualized Me31B-GFP at high time resolution. We observed Me31B-GFP particles moving with an average velocity of approximately 0.25 $\mu\text{m/s}$ (Fig. 2b,f, Supplementary Movie 2,3), significantly slower than RNA particles (Fig. 2f). This suggests that Me31B is in continual flux as small dynamic particles that leave or join larger Me31B rich P bodies. We then tested the degree of exchange of Me31B in and out of P bodies using Fluorescent Recovery After Photobleaching (FRAP) experiments. We found that Me31B::GFP recovers to approximately 50% of total fluorescence with a half time of approximately 4 minutes after photobleaching (Fig. 2g). In contrast, control experiments using freely diffusing GFP fused to a nuclear localization signal (nlsGFP) recovered over 90% of total fluorescence in less than 5s ($n=5$, data not shown). We conclude that Me31B::GFP is not freely diffusible, but instead is maintained by relatively slow exchange of dynamic particles that move independently of *grk* and *bcd* mRNA.

To begin to understand how this organization could influence translation, we characterized the distribution of key translation components in the oocyte at mid-oogenesis (Fig. 3a-g, Supplemental Fig. 3c-l). Our results show that many factors involved in mRNA localization and translational regulation including Exuperantia (Exu), Trailer hitch (Tral) and Growl/Lost do not colocalize with *grk* mRNA but display the same distribution as Me31B (Supplemental Fig. 3c-e, Fig. 1f). Similarly, Cup and Eukaryotic initiation factor 4E (eIF4E) colocalize with P bodies (Supplemental Fig. 3g-k). Furthermore, using IEM, we show that Me31B, Heterogeneous nuclear ribonucleoprotein at 27C (Hrb27C/Hrp48/p50) and the mRNA degradation enzyme Decapping protein 2 (Dcp2), a hallmark of P bodies in yeast and mammalian cells⁹, are significantly enriched in the P body core when compared to the surrounding cytoplasm, suggesting that the P body core is translationally silent (Fig. 3a-c,e, Supplemental Fig. 3f).

In line with the role of P body in translational inhibition, previous work showed that P bodies lack ribosomes, as judged by morphological analysis and by a GFP tagged ribosomal component^{8,18}. We confirmed this by labeling endogenous ribosomes by IEM using an antibody against a specific ribosomal protein (Fig. 3d,e, Supplemental Fig. 4, Supplemental Fig. 2d and Supplemental methods). We found that 91% of ribosomes are not associated with P bodies. Interestingly, the 9% of ribosomes associated with the P bodies are almost exclusively enriched at the P body edge (Fig. 3e), and some of them are on ER strands closely apposed to P bodies (Fig. 3d). We conclude that the core of oocyte P bodies cannot

support translation, whereas the edge of P bodies could support translation, for instance, of *grk* mRNA.

To further test this, we examined the distribution of the translational activator Orb/CEPB. Although Orb has not been shown to bind directly to *grk* mRNA, it is known to be required for *grk* mRNA localization and translation¹⁹. Our results show that Orb is enriched at the P body edge (Fig. 3e,f, Supplemental Fig. 2b), suggesting that the edge of P bodies could be an Orb mediated site of translational activation. We also determined the distribution of Squid (Sqd) in P bodies, as it is required to maintain *grk* mRNA localization and we previously showed that it is present in P bodies and within *grk* RNP transport particles⁷. We used an anti-GFP antibody to detect Sqd::GFP and showed that Sqd is enriched 3.5 times at the P body edge compared to its core (Fig. 3e,g). We conclude that in the oocyte, there is a distinct edge region of the P body that is competent for translation while the core is a site of translational repression. This is the first evidence suggesting a relationship between the P body stratified organization and the translational fates of distinct mRNAs.

To test directly whether the edge of P bodies is also the site of *grk* translation, we covisualized *grk* mRNA and protein at the ultrastructural level. Our results show that, at steady state, the intracellular pool of endogenous Grk protein is found in the early secretory pathway, within the ER and tER-Golgi units. This distribution is in line with the fact that Grk protein is first synthesised as a transmembrane protein, processed and secreted into the intracellular space between the oocyte and dorsoanterior follicle cells (Fig. 4a)¹². Although Grk protein is largely found at a different location from its mRNA, we found a small amount of Grk protein is also present on rough endoplasmic reticulum (RER) at the edge of P bodies (Fig. 4a,b, Fig. 3d) where *grk* mRNA is docked (Fig. 1b-d). We also observed *grk* mRNA transport particles lacking Grk protein (see arrowhead in Fig. 4b) that are present at the dorsoanterior corner but are not docked to P bodies. Given that *grk* mRNA is repressed during transport^{5,20}, we favour the interpretation that translational activation of *grk* mRNA occurs when transport particles dock at the edge of P bodies from where Grk protein enters the secretory pathway.

At steady state, the majority of Grk protein is located in the secretory pathway¹². Therefore, it is difficult to determine where *grk* mRNA is first translated. To overcome this, we injected a mixture of AlexaFluor labeled RNA and unlabeled RNA encoding a full length Grk protein into *grk* mRNA null oocytes⁵ (Fig. 4c,d). Despite potentially not being subject to the normal translational repression while being transported, our results show that a pulse of Grk protein translation is colocalized with *grk* RNA, and both were interdigitated with Me31B (Fig. 4c, arrowheads). We interpret these results as showing that *grk* mRNA is translated at the P body edge.

Precise regulation of the dosage level of Grk protein in the oocyte is essential for correct dorsoventral patterning²⁰. To test whether an excess of *grk* mRNA is translationally repressed, we injected varying amounts of *grk* RNA and characterized its distribution after localization. Injection is a well established assay for monitoring RNA localization^{3,21}. Despite being additional to endogenous *grk* mRNA, injected *grk* RNA assembles into transport particles that become localized at the DA corner²². We show that moderate

concentrations of injected *grk* RNA (~120 ng/μl) localizes and interdigitates with Me31B, at the edge of the P bodies (Supplemental Fig. 5a, b). In contrast, injecting four fold more *grk* RNA leads a proportion of RNA localizing to the core of P bodies (Fig. 4e, Supplemental Fig. 5c,d). We conclude that an excess of injected *grk* RNA is directed to the P body core.

To test whether an excess of endogenous *grk* mRNA behaves like injected RNA, we used the UAS-Gal4 system to drive *grk* over-expression. We confirmed that over expression occurred with anti-Grk antibodies and *grk* FISH (Supplemental Fig. 5e,f), and found dorsoventral patterning defects in eggs to varying degrees²³. We used ISH-IEM to determine where over expressed *grk* mRNA is localized and found a clear increase in the relative proportion of *grk* mRNA found in the core of P bodies when compared to wild-type (Fig. 4f, Supplemental Fig. 5g,h). Together, these results support a model where excess *grk* mRNA is directed into the core of the P body, which lacks ribosomes and maintains translational repression.

To further test the relationship between P body sub-localization and differential translation, we analyzed translationally repressed *bcd* mRNA in oocytes and translationally active *bcd* mRNA in embryos⁶. At stage 9 of oogenesis, a population of *bcd* is enriched in the P body core (Fig.1), whereas the remainder is in highly dynamic particles that are known to also be translationally repressed by a mechanism that is not well understood. At stage 14 of oogenesis, when *bcd* mRNA is known to be anchored and repressed^{6,24}, all of it colocalizes with Me31B (Supplemental Fig. 5i,j). In contrast, in early embryos, at a time when it is translated, *bcd* mRNA is outside of P bodies (Fig. 5a). It is not known when and how *bcd* translation is activated in embryos, but we hypothesize that this may be related to egg activation, a major transition point between oocytes and embryos.

We previously showed that *bcd* mRNA is released from its anterior cytoskeletal anchor by egg activation²⁴. To test whether egg activation is also the trigger for *bcd* mRNA redistributing outside the P bodies, we covisualized labeled P bodies and *grk**MS2, before and after activating eggs *in vitro*. We found that upon egg activation, Me31B (Fig. 5b) and Tral (Fig. 5c) become dispersed, and results in a loss of colocalization between *bcd* mRNA and Me31B (Fig. 5d,d'). Although our data does not address directly whether the translational activation of *bcd* mRNA is caused by P body disassembly, we previously showed that in *sarah* mutant embryos, where activation does not occur, *bcd* mRNA is not released from the anterior or translated^{24,25}. Therefore, we propose that translational activation of *bcd* mRNA results from the disassembly of P bodies upon egg activation.

Our results are the first to demonstrate the stratification of P bodies and suggest how localized transcripts could be differentially translated by distinct associations with P body zones. We propose a model for the spatio-temporal regulation of the translation of developmentally important mRNAs in the oocyte (Fig. 5e). Translationally repressed RNP transport particles associate with the edge of P bodies where anchoring and translation activation factors, such as Sqd and Orb, are enriched. We previously showed that disrupting Sqd function causes a defect in anchoring of transport particles at the dorsoanterior P bodies⁷. Instead of docking at the dorsoanterior corner, *grk* transport particles are in continuous flux along the anterior and *grk* is inappropriately translated⁷. We therefore favor

a model in which *squid* is required for translational repression of *grk* mRNA during its transport, and for anchoring the transcript at the P body edge. In this model, Orb is also present at the edge of the P body and is required for translational activation of *grk* mRNA. RNA, such as *grk*, that is excluded from the core of the P body is translationally activated at the edge of the bodies. In contrast, *bcd* and probably other transcripts, that enter the ribosome-depleted core of P bodies remain translationally repressed. Furthermore, the finding that excess *grk* RNA leads to an increase in the proportion of *grk* in the P body core, leads us to speculate that this is a mechanism for regulating Grk protein levels.

P bodies are one of several related electron dense and RNP-rich cellular structures²⁶ that include stress granules²⁷, the *Xenopus* mitochondrial cloud²⁸, the zebrafish Balbiani body²⁹, and nematode P-granules³⁰. The dynamic nature of the protein and mRNA components of these structures has not often been studied in detail, except in *C.elegans* where P-granules have been shown to behave in a fluid like manner³¹. Our observation of highly dynamic independent movements of a P body protein and mRNA provides a general molecular basis for the fluid like behavior of P-granules and related granules. There has been considerable debate about whether P body formation is essential for mRNA decay and translational repression or a consequence of these events³²⁻³⁵. The consensus has been that P body formation is essential for mRNA decay and translational repression³²⁻³⁵ and our work highlights another function for P bodies in translational regulation of key transcripts through partitioning between the core and edge of the bodies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Differential association of *bcd* and *grk* with P bodies

(a-d) mRNA detection at the dorsoanterior corner (for orientation, see Fig. 2a) by ISH-IEM on wild-type (WT) ultra-thin frozen sections of stage 9 oocytes. (a) *bcd* mRNA (5nm, green circles) is present both inside and at the edge of electron dense P bodies (dashed black line). Gold particles here cluster due to the use of a bridging antibody. (b) *grk* mRNA (15nm, red circles) is enriched at the edge of P bodies (dashed black line). (c) *bcd* mRNA (5nm, green circles) and *grk* mRNA (15nm, red circles) can associate with the same P body but *bcd* is enriched inside. (d) *grk* mRNA (15nm, red circles) docks at the edge of the P body (black dashed box magnified, inset bottom right), just outside of the Me31B dense core region (5nm gold), while *grk* transport particles, as described in Delanoue et. al., 2007⁽⁷⁾, are detected in the cytoplasm at a short distance from the P body (15nm, red dashed circles). (e) mRNA density (gold/μm²) in the P body sub-regions when compared to the surrounding cytoplasm. For comparison, randomized particles were analyzed in an identical way to RNAs. Error bars show SEM of gold density per scan (*grk* n=13, *bcd* n=11). (f,g) Fixed Me31B::GFP stage 8/9 expressing oocytes imaged using the OMX structured illumination super-resolution mode (3D-SIM) with double labeling of either (f) *grk**mCherry, that mainly interdigitates with Me31B (green) while showing some colocalization at the edge (yellow) or (g) *bcd**RFP *grk* mRNA (red), that shows significant colocalization (yellow) with Me31B (green). Scale bars, 200nm (a-d); 0.5μm (f, g).

Figure 2. Dynamics of *grk*, *bcd* and Me31B particles in live oocytes

(a) Low magnification wide-field image of the DA corner of a stage 8-9 egg chamber expressing Me31B::GFP. (b) Me31B::GFP expressing oocytes show small faint dynamic particles of Me31B (red arrowheads) moving between and fusing together with other often larger, bright and static (dashed cyan circles) Me31B bodies. (Supplemental Movie 1, 2). (c,d) *grk***mCherry* and Me31B::GFP expressing oocytes show *grk* mRNA particles (white arrowheads) moving independently of Me31B (Supplemental Movie 3). (d) Dynamic particles of *grk* (white arrowhead) are visualized docking and remaining on the edge of Me31B rich zones. Other small *grk* particles are seen in association with the Me31B throughout the time course (dashed cyan circles) (Supplemental Movie 4). (e) *bcd**RFP and Me31B::GFP expressing oocytes show *bcd* particles (white arrowheads) moving independently of Me31B. (Supplemental Movie 5). (f) Average particle velocities for dynamic Me31B ($n=30$), *grk* ($n=37$) and *bcd* ($n=31$) particles in $\mu\text{m}/\text{second}$ $\pm$ SEM. P-values from student's t-tests (two tails), $P < 0.001$. (g) Fluorescence Recovery After Photobleaching of Me31B::GFP at the DA corner shows recovery to approximately 50% of total fluorescence with a half time of 4 minutes ($n=5$). NC, Nurse Cell; N, nucleus. Scale bars, $10\mu\text{m}$ (a); $2\mu\text{m}$ (b-e); $20\mu\text{m}$ (g).

Figure 3. Oocyte P bodies exhibit zones differentially enriched in RNA associated proteins (a-d,f,g) IEM localization of RNA associated proteins in stage 9 oocytes at the DA corner. Protein in the core of the electron dense P bodies indicated by green circles, at the edge of P bodies by red circles and in the cytoplasm by blue circles. (a) WT egg chamber, anti-Me31B (15nm) enriched inside P bodies. (b) Hrb27C (5nm) predominantly inside of P bodies. (c) Dcp2 (10nm) inside and at the edge of P bodies. (d) WT egg chamber, anti-Ribo 490 (10nm) shows ribosomes predominantly in the cytoplasm some at the edge but mostly excluded from inside of P bodies. The pool of cytoplasmic ribosomes corresponds to polysomes and those present on the ER membrane (Rough ER), some of which is present near the edge of a P body (black dashed box, inset: red arrowhead). Smooth ER is detected inside of the P body (inset: black arrowhead). (e) Graph showing protein concentration in gold/μm² in the cytoplasm, edge of P bodies and inside of P bodies; proteins are organized into three distinct categories. Error bars are ± **standard deviation** (between n=10-15 scans in each case). (f) WT egg chamber, anti-orb (15nm) enriched at the edge of P bodies. (g) Sqd-GFP in Sqd::GFP expressing egg chamber using anti-GFP (10nm) and anti-Dhc (15nm, yellow circles). Sqd is enriched inside compared to at the edge of P bodies. Scale bars, 200nm (a-d,f,g).

Figure 4. RNA translation fate is regulated through association with P bodies

(a) IEM using anti-Grk (15nm, yellow circles) on a WT stage 9 egg chamber shows Grk protein highly enriched in the tER-Golgi unit (yellow dashed box) and detected on ER (black arrowhead) at the edge of a P body (black dashed line). (b) ISH-IEM detection of *grk* mRNA and Grk protein on a WT stage 9 egg chamber shows *grk* mRNA (15nm, red circles) mostly present at the edge of the P body (black dashed line) and Grk protein (5nm, yellow circles) on a tER-Golgi unit (yellow dashed box) but also present at the edge of the P body (black dashed line). Note the *grk* mRNA transport particle (dashed red circle) does not contain any detectable Grk protein (black arrowhead). (c) Injection of *in vitro* synthesized *grk* RNA (mixed solution of approximately one part Alexa dye labeled and 4 parts unlabeled) into a *grk* null egg chamber expressing Me31B::GFP. Images shown are a 4μm projection. Localization and translation was allowed for 40 minutes before the egg chamber was fixed and labeled with anti-Grk. (c, c'') Grk protein (green) and (c, c''') *grk* RNA (red) colocalize, but do not colocalize with (c, c') Me31B (blue). Instead, *grk* RNA and protein interdigitates with Me31B (arrowheads). (d-d'') Low magnification of panel c. Injected *grk* RNA is localized to the dorsoanterior corner where it is translated. (e) IEM on a WT stage 8/9 egg chamber injected with a high concentration of *grk* RNA Biotin (500 ng/μl). Injected RNA (15nm, red circles) is detected at the core of and at the edge of the P body (dashed black line). (f) IEM on a stage 8/9 egg chamber over-expressing *grk* mRNA using the UAS-Gal4 system. *grk* mRNA (15nm) is enriched at the core of and at the edge of the P body

(dashed black line). FC, follicle cell; TP, transport particle; ER, endoplasmic reticulum; N, nucleus. Scale bars, 200nm (**a,b,e,f**); 3 μ m (**c**); 10 μ m (**d**).

Figure 5. RNA association with P bodies in the early embryo

(a) Anterior region of a live early embryo expressing *bcd**RFP and Me31B::GFP. The majority of *bcd* mRNA is detected outside of Me31B labeling, sometimes associating with the edge (arrowhead). (b,c) Time points from a stage 14 egg chamber expressing (b) Me31B::GFP or (c) Tral::YFP, prior to and following activation. Activation buffer added at t = 0 minutes. Immediately after activation, Me31B (or Tral) labeled P bodies disperse. (d) Stage 14 egg chamber expressing *bcd**RFP and Me31B::GFP (d) Before treatment with activation buffer and (d') 10 minutes after activation buffer is applied. Pre-activation, *bcd* mRNA and Me31B colocalize at the anterior (arrowhead). Immediately after activation, Me31B disperses and *bcd* no longer colocalizes with Me31B (arrowhead). (e) Model of how P bodies regulate differential translation of *bcd* and *grk* mRNA at the DA corner in a stage 8/9 oocyte. *grk* mRNA transport particles move independently of Me31B. *grk* associates at the edge of the P body where enrichment of Sqd is involved in anchoring, Orb activates translation and ribosomes support translation. *grk* is translated on rough ER at the edge of P bodies and Grk protein is trafficked through the secretory pathway (tER-Golgi unit) before being secreted. *bcd* mRNA transport particles move independently of Me31B and associate with the core of the P body where translation is not supported. Dynamic Me31B, Exu and Tral particles assemble and maintain the P bodies. Excess *grk* RNA is targeted into the core of P bodies where it is translationally repressed and likely degraded. FC, follicle cell. Scale bars, 5μm (a,d); 20μm (b,c).