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**The 5' – 3' exoribonuclease Pacman is required for normal male fertility and is dynamically localised in cytoplasmic particles in *Drosophila* testis cells.**

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Running title: Role of Pacman in *Drosophila* Spermatogenesis.

Key words: P-bodies, Xrn1, RNA stability, spermatogenesis, RNA degradation

## Synopsis

The exoribonuclease Xrn1 is widely recognised as a key component in the 5'-3' RNA degradation pathway. This enzyme is highly conserved between yeast and humans and is known to be involved in RNA interference, degradation of microRNAs as well as RNA turnover. In yeast and human tissue culture cells, Xrn1 has been shown to be a component of P-bodies which are dynamic cytoplasmic granules where RNA degradation can take place. In this paper we show for the first time, that Pacman, the *Drosophila* homologue of Xrn1, is localised in cytoplasmic particles in *Drosophila* testis cells. These particles are present in both the mitotically dividing spermatogonia derived from primordial stem cells and in the transcriptionally active spermatocytes. Pacman is co-localised with the decapping activator dDcp1 and the helicase Me31b (a Dhh1 homologue) in these particles although this co-localisation is not completely overlapping, suggesting that there are different compartments within these granules. Particles containing Pacman respond to stress and depletion of 5' -3' decay factors in the same way as yeast P-bodies therefore are likely to be sites of mRNA degradation or storage. Pacman is shown to be required for normal *Drosophila* spermatogenesis, suggesting that control of mRNA stability is crucial in the testis differentiation pathway.

## Introduction

The control of RNA stability is a crucial element of gene regulation in all organisms. There is increasing evidence that specific, timed transcript degradation is critical for regulation of many cellular processes, including early development and RNA interference. The importance of RNA stability in developmental processes is revealed by the phenotypes of animals or plants where the expression of genes involved in RNA stability has been disrupted. For example, mice deficient for TTP, an RNA binding protein regulating stability of RNAs such as GMCSF and TNF, develop a systemic inflammatory syndrome with autoimmunity and myeloid hyperplasia (bone marrow overgrowth) [1]. In *Drosophila*, mutations in the 3' – 5' deadenylase *ccr4* (*twin*) result in a level of sterility and maternal effect lethality in females [2]. Furthermore, *C. elegans* adults carrying mutations in *dicer* (*dcr-1*), a double-stranded endoribonuclease which plays a key role in the first steps of the RNA interference pathway, are sterile and have defects in developmental timing [3, 4]. Finally, *Arabidopsis* mutant for DST1, which regulates the stability of RNAs such as CCL and SEN1, have defects in their circadian rhythm [5]. Therefore transcript degradation during development can be selective and also modulated suggesting a little studied layer of control of gene expression during development.

One of the key ribonucleases in the 5' – 3' mRNA degradation pathway is the exoribonuclease Xrn1. This enzyme is known to be a processive 5' – 3' exoribonuclease which degrades mRNA after they have been de-capped [6-8]. Xrn1 is extremely well conserved in all eukaryotes, with homologues in humans (*xrn1*), *Drosophila* (*pacman*) and *C. elegans* (*xrn-1*) [9, 10]. Indeed we have previously shown that *Drosophila* Pacman has exonuclease activity in-vitro and that it can complement a null XRN1 mutation [9-11]. Xrn1 is not only involved in the normal decay of mRNA but is required the major pathway in nonsense-mediated decay [12], RNA interference [13] and degradation via microRNAs [14]. Mutations in *xrn1* in yeast or silencing of *xrn-1* in *C. elegans* leads to phenotypic defects suggesting that this enzyme is required for normal cellular processes [6, 8, 9, 15]. Our recent work has also shown that mutations in *pacman* in *Drosophila* result in defects in dorsal and thorax closure and reduced survival after wound healing [16].

Recent exciting work has shown that 5' – 3' degradation of cytoplasmic mRNAs seems to take place in specific particles known as processing bodies (P-bodies) [17-19]. In both yeast and human cells, P-bodies have been shown to harbour many enzymes of the basic decay machinery including the decapping complex and the exoribonuclease Xrn1. Strong evidence that P-bodies are sites of normal mRNA decay rather than merely storage sites for RNA decay factors is that they accumulate mRNA decay intermediates [17, 20] although mRNA degradation may also take place in sub-microscopic particles [21]. In mammalian cells P-bodies are not only involved in normal mRNA turnover but have also been reported to be the sites of nonsense-mediated decay and miRNA-induced translational silencing [22, 23]. P-bodies are dynamic structures that can vary in size and number according to stress conditions or the availability of untranslated RNAs [24, 25]. Although P-bodies have been well studied in yeast and human tissue culture cells, they have rarely been observed in natural tissues. Their relevance to developmental processes is also unclear.

In this paper, we have examined the function of the exoribonuclease *pacman* (*pcm*) in *Drosophila* testis. We have chosen testis as a model system because they undergo a well defined differentiation pathway, remarkably similar to spermatogenesis in mammals [26]. In both flies and mammals, cells (spermatogonia) produced from self-renewing germ cells first mitotically divide to form spermatocytes and then, after a period of growth and

transcription, shut off the transcriptional programme, enter meiotic divisions and differentiate into spermatids. Post-transcriptional pathways are highly regulated during the spermatogenesis process with many transcripts being synthesised in primary spermatocytes and then translationally repressed until long after meiotic divisions [27]. The contribution of mRNA stability to spermatogenesis is as yet unclear.

This paper analyses the effect of the 5' – 3' exoribonuclease *pacman* on male fertility in the fruit-fly *Drosophila melanogaster*. We show that Pacman is required for normal testis development and male fertility. We also demonstrate that Pacman is located in cytoplasmic particles akin to P-bodies in both mitotically dividing spermatogonia and transcriptionally active spermatocytes. These particles also include the decapping activator dDcp1 and the helicase Me31b. These P-bodies, like those in human tissue culture cells, increase in size and number upon stress. Therefore we show, for the first time, that an exoribonuclease is crucial to normal male spermatogenesis.

### Materials and Methods

Fly stocks were cultivated on standard media at 25°C in uncrowded conditions. The wild-type stock was Oregon R (Bloomington Stock Centre). The transgenic stock P{w+; YFP-dDcp1} was provided by M.D. Lin [28]. The *pcm* mutants were generated by P-element excision and have been described previously [16]. Molecular characterisation of the *pcm*<sup>3</sup> and *pcm*<sup>5</sup> alleles showed that the *pcm*<sup>3</sup> allele comprises a deletion of 1522 bp resulting in a frame-shift mutation. The resulting mutant protein is a C-terminal truncation of 197 amino-acids relative to wild-type plus an extra 46 amino-acids encoded from intergenic sequences; the 3' UTR was also deleted. The *pcm*<sup>5</sup> allele is an intragenic deletion which removes 516 base pairs, resulting in a frame-shift. The *pcm*<sup>5</sup> protein is C-terminally truncated by 348 amino-acids compared to wild-type, with a 29 amino-acid extension encoded from out-of-frame *pcm* sequences. The 3' UTR is intact in this mutant. The wild-type protein is 1612 amino-acids.

### Measurement of male fertility.

The fertility of *pcm* mutant males was tested by crossing (at 25 °C) individual virgin males (control, *pcm*<sup>3</sup> and *pcm*<sup>5</sup>) to individual wild-type virgin females. 25 vials per strain were set up. The flies were left to mate for 2 days and then removed from the vials. F1 generation offspring were counted for 8 days after the first offspring hatched. The observed differences between the control and *pcm*<sup>3</sup> and control and *pcm*<sup>5</sup> were statistically significant in both cases (Mann-Whitney test,  $W=415.5$ ,  $P<0.0001$  and  $W=389$ ,  $P<0.0001$ , respectively).

### Measurement of sperm number.

Seminal vesicles were dissected from 3 day old virgin males ( $n=15$ , at 25 °C) in a drop of phosphate buffered saline (PBS) and transferred to a fresh drop of PBS. The seminal vesicles were then ruptured and the sperm inside allowed to dissociate. The preparations were left for 10 min and then allowed to dry. The time allowed for dissociation and drying ensured that the sperm were well spread out. The slides then fixed with 20  $\mu$ l 4% paraformaldehyde solution in HEPES buffer and stained with DAPI. Samples were mounted in 85% glycerol with 2.5% n-propyl gallate and cover slips were sealed with nail varnish. Sperm were then viewed at X40 power with a Chroma long pass DAPI filter using a Leica fluorescent microscope. A video camera and monitor were used to view the slides and the sperm were counted. The observed differences between the control and *pcm*<sup>3</sup> and also the control and *pcm*<sup>5</sup> mutants were statistically significant in both cases (Mann-Whitney test,  $W=0$ ,  $P<0.0001$  and  $W=0$ ,  $P<0.0001$ , respectively).

### Measurements of testes.

Testes were dissected from 2 day old virgin males ( $n=20$ , at 25 °C) in Ringer's solution (130 mM NaCl, 4.7 mM KCl, 1.9 mM CaCl<sub>2</sub>, 10 mM HEPES buffer, pH 6.9) and mounted in Ringer's solution on a microscope slide without a coverslip. The testes were visualized with light microscopy at X20 power and the length and width (200  $\mu$ m from the tip of the testes) of the testes were measured with Zeiss AxioVision imaging system software. The observed differences for the length and width of the testes between the control and *pcm*<sup>3</sup> and also the control and *pcm*<sup>5</sup> were statistically significant in both cases (for the length of the testes: Mann-Whitney test,  $W=255$ ,  $P<0.025$  and  $W=361$ ,  $P<0.0001$ , respectively; for the width of the testes: Mann-Whitney test,  $W=445$ ,  $P<0.0001$  and  $W=283$ ,  $P<0.0001$ , respectively).

### Western blotting and co-immunoprecipitation from testes extract.

SDS-PAGE and Western blotting was performed essentially as described [29] with binding of antibodies being detected using a ECL Western blot reagent kit (Amersham). Primary polyclonal anti-PCM antibody (raised in rabbit) [16] was used at 1:2000. We are confident that this antibody (raised to the 54kD C-terminal portion of the protein) can detect the truncated mutant *pcm* proteins because it can pull down PCM from testis extracts (Figure 4, panel L). The monoclonal anti-actin antibody (Sigma) was used at 1:10000 and primary antibody anti-dDcp1 (raised in rabbit), provided by J. Wilhelm, was used at a dilution of 1:2000. The secondary antibodies were monoclonal anti-rabbit HRP conjugated antibody (Sigma) and monoclonal anti-mouse HRP conjugated antibody (Sigma), both used at 1:80000.

Testes dissected from 0-1 day old males were homogenized in lysis buffer (50 mM Tris-HCl pH 7.5-8.0, 0.5% Triton X-100, 150 mM NaCl, protease inhibitors) (140 testes, 500  $\mu$ l buffer used), then cleared by centrifugation (10,000 rpm for 10 min at 4°C). 40  $\mu$ l was retained as the "input" sample, the remainder was pre-cleared with protein G-sepharose (Roche), then incubated with rabbit anti-PCM and precipitated with protein G-sepharose (at 4°C). Beads were washed, then bound proteins were eluted by boiling in SDS sample buffer. For the negative control wild type testes were immunoprecipitated without any anti-PCM antibody.

### Immunofluorescence

For immunofluorescence, testes were dissected from 0-1 day old males in Ringer's solution, with testes from about ten males being used per slide. The testes were transferred to a 20  $\mu$ l drop of Ringer's solution on a poly-L-lysine-treated slide and cut open with tungsten needles. Paraformaldehyde (20  $\mu$ l of 4% solution) in HEPES buffer was added and the testes were left to fix for 15 minutes at room temperature. Testes were squashed by adding a cover slip, the slide was dipped in liquid nitrogen and the cover slip was removed with a scalpel. Testes were then stored in PBS+0.1% Tween-20 (PBS-T) until all the samples had been prepared. Samples were blocked for 30 minutes with PBS-T+10% FCS, then incubated with primary antibody diluted in blocking solution overnight at 4°C. Testes were rinsed once then washed four times for 10 minutes each in PBS-T. Testes were incubated with secondary antibodies diluted in blocking solution for 2 hours at room temperature. Samples were washed as before and mounted in 85% glycerol with 2.5% n-propyl gallate. Cover slips were sealed with nail varnish and the samples were imaged using a Leica TCS SP 2UV confocal microscope.

Primary antibodies used were rabbit anti-PCM, rabbit anti-dDcp1 (provided by J. Wilhelm) and mouse anti-Me31B (provided by A. Nakamura). For immunohistochemistry, primary antibodies were used at a dilution of 1:500, 1:1000, and 1:1000, respectively. Secondary antibodies used were Cy-3-conjugated monoclonal mouse anti-rabbit IgG, Cy-5-

conjugated goat anti-mouse IgG, FITC-conjugated goat anti-rabbit IgG (Jackson Immuno Research). The secondary antibodies were used at a dilution of 1:200. The size of the cytoplasmic particles were measured using ImageJ software [30]. In each case the size of particles were measured for 5 different areas (35  $\mu\text{m}$  x 35  $\mu\text{m}$ ) to ensure repeatable results. The observed differences between the cases were statistically significant according to Mann-Whitney test. The difference between the sizes of the cytoplasmic particles in *pcm*<sup>5</sup> mutants under normal and stress conditions as detected using the dDcp1 antibody were rather low and the results of the Mann-Whitney test were inconclusive ( $W=236$ ,  $P=0.091$ , which is  $>0.05$  but  $<0.1$ ); therefore we also used the exact permutation test. The percentage of co-localisation was calculated using ImageJ software. In each case the percentage of co-localisation was determined for 3 areas (25  $\mu\text{m}$  x 25  $\mu\text{m}$ ) to ensure repeatable results.

## Results

### The 5' – 3' exoribonuclease *pacman* affects male fertility in *Drosophila*

In order to study the effect of the 5' -3' exoribonuclease *pacman* on male fertility we examined males carrying mutations in the *pacman* gene. These mutations had been previously constructed using P-element excision of a P-element transposon located 584nt downstream of the *pcm* polyA site, between *pcm* and the convergent gene *Nat1* on the X-chromosome at position 18D [16]. Two of the strongest alleles (*pcm*<sup>3</sup> and *pcm*<sup>5</sup>) were used in this study (Figure 1, panel A). Molecular characterisation of these alleles showed that both encoded truncated proteins. The *pcm*<sup>3</sup> allele was the result of a deletion from the P-element insertion site into the 3' end of the *pcm* gene and resulted in a C-terminal truncation of 197 amino-acids relative to wild-type. The *pcm*<sup>5</sup> allele results from an intragenic deletion and encodes a protein C-terminally truncated by 348 amino-acids (see Methods for further details). Using a polyclonal antibody which specifically detects PCM [16] in Western blotting experiments we showed that testes dissected from hemizygous *pcm*<sup>3</sup> males produced truncated protein at levels significantly lower than wild-type whereas *pcm*<sup>5</sup> animals expressed very low levels levels of *pcm* protein (Figure 1, panel B and C).

We next tested the effect of these *pcm* mutant alleles on male fertility by mating individual males to 3-5 day old OregonR wild type virgin females and counting the offspring. The *pcm*<sup>5</sup> males were seriously compromised in their fertility producing only 47% of the progeny of control males at 25°C. (Figure 2, panel A). Examination of testes from these mutant males showed that the mutant testes from 1 day-old *pcm*<sup>5</sup> males were much shorter and thinner than controls (Figure 2, panels B and D). Measurements of the length and width of these testes also showed that they were smaller than controls (Figure 2, panels C, D and G). Male *pcm*<sup>3</sup> flies were intermediate in numbers of offspring and size of testes between *pcm*<sup>5</sup> and controls (Figure 2, panels A, C, D and F). These smaller testes produced fewer sperm (68% of wild-type in the case of *pcm*<sup>3</sup>; 52% of wild-type for *pcm*<sup>5</sup>, (Figure 2, panel B) which would account for the lower fertility of the *pcm* mutants.

### Pacman is localised in cytoplasmic particles in wild-type testes

To start to understand how the exoribonuclease *pcm* might affect male fertility, we first examined the expression and localisation of *pcm* protein in *Drosophila* testis. Using our anti-PCM antibody, we showed that PCM is well expressed at the tip of the testis, in the spermatogonia where the initial mitotic divisions take place (Figure 3, panel A) [26]. Closer examination revealed that PCM is located in foci in these cells (Figure 3, panel B). To confirm that PCM is located in cytoplasmic granules in living cells, we generated a UAS-GFP-PCM transgenic which expresses GFP fused in frame to the N-terminus of PCM under the control of the UAS enhancer. When this GFP-PCM is expressed in testes using the *bam*-GAL4 driver, GFP-PCM also accumulates in particles (Figure 3, panel C).

However in bam-Gal4, UAS-GFP-PCM flies the GFP-PCM expression also appears in the fusome, a membranous organelle that allows endoplasmic reticulum connectivity between cells [31]. This is most likely to be because it is over-expressed from the foreign promoter. The expression of PCM in spermatogonia suggests that it is required for the amplification or maintenance of these dividing spermatogonia prior to their entry into pre-meiotic S-phase as primary spermatocytes.

We then examined the localisation of PCM in primary spermatocytes, which are cells that do not divide but undergo massive transcription and growth. In these cells, PCM is located in discrete, cytoplasmic foci as detected in both GFP-PCM flies (Figure 3, panel D) and immunocytochemistry experiments (Figure 3, panel E). The staining of granules in testis from transgenic flies expressing GFP-PCM co-localises with PCM antibody staining (Figure 3, panel F). These experiments show that PCM is also required at this stage of differentiation.

This is the first time that cytoplasmic foci that may be related to P-bodies have been seen in *Drosophila* testis. These particles are reminiscent of Xrn1 particles in mouse cells under conditions where the cells are not stressed, and native levels of protein (rather than protein expressed from a foreign promoter) are observed. In human tissue culture cells such as HEK293 cells, P-bodies can also be present in unstressed cells [24]. This data shows that PCM is dynamically localised in testis cells and suggests that these particles could be *Drosophila* P-bodies.

#### **Pacman is co-localised with dDcp1 and Me31B in testis cells**

In both yeast and human tissue culture cells P-bodies have been shown to contain enzymes required for decapping of mRNA (dDcp1 and dDcp2) as well as the helicase Me31B/Dhh1/p54 [17-20, 32, 33]. In both human and yeast cells, Dcp1 is generally regarded as a marker for P-bodies [34]. To determine whether the PCM foci seen in testis cells might be P-bodies, we investigated whether dDcp1 is located in cytoplasmic granules and if PCM is co-localised with dDcp1.

Use of both an anti-dDcp1 antibody and a dDcp1-YFP transgenic show that dDcp1 is localised in cytoplasmic granules in both spermatogonia and primary spermatocytes (Figure 4, panel A and B). Co-localisation experiments showed that dDcp1 is largely, but not completely co-localised with PCM in spermatocytes in that 95% of PCM protein visualised is co-localised with dDcp1 (Figure 4, panels C-E).

Another protein known to be critically important in P-body formation in *S. cerevisiae* is the RNA helicase Dhh1. This enzyme is highly conserved with the known homologue in *Drosophila* being Me31B [35]. Using an antibodies to Me31B, dDcp1, and PCM we showed that Me31B co-localises with dDcp1 (Figure 4, panels I-K) and Me31B co-localises with PCM (Figure 4, panels F-H) in spermatocytes (95% in both cases). Close examination of these particles revealed that co-localisation of Me31b and dDcp1 was almost complete, whereas both of these were partially co-localised with PCM (see insets in panels E, H and K). This suggests a structure to these particles which has not previously been observed. The co-localisation between PCM, dDcp1 and Me31B strongly suggest that these particles are akin to P-bodies.

To determine whether PCM physically interacts with dDcp1 we carried out immunoprecipitation experiments. Figure 4 panel L shows that our PCM antibody can co-precipitate dDcp1 protein from both wild-type and mutant testes extracts (no cross-linking procedures were used). Therefore PCM can bind to dDcp1, suggesting molecular and functional interactions between the two proteins.

### Pacman mutations affect the size and numbers of testis P-bodies

In the yeast *S. cerevisiae*, cells mutant for Xrn1 are blocked in 5' – 3' exonuclease degradation (*xrn1Δ*) show dramatic increases in size and number of P-bodies [17]. If these testis granules are the sites where mRNA degradation takes place, we would therefore expect the size of P-bodies to increase when the Xrn1 homologue Pacman is depleted. To test this prediction we first immunostained hand-dissected testes using our PCM antibody. In *pcm<sup>5</sup>* mutants, only diffuse, low intensity staining was seen with no cytoplasmic foci visible (Figure 5, panel C). This result is consistent with our Western blotting experiments (Figure 1, panel B) where no PCM band was visible. The *pcm<sup>3</sup>* mutants showed diffuse, faint staining with few obvious cytoplasmic particles (Figure 5, panel B). Note that this staining in panels B and C was visualised using a higher power of the laser than the other panels. Therefore, as expected, these hypomorphic mutations result in a reduction of staining in these mutants. In addition, in the case of the *pcm<sup>3</sup>* mutation which encodes a C-terminal truncation of PCM (Figure 1, panel A), these experiments also suggest that the C-terminus of PCM may be important in localising PCM to P-bodies. The C-terminus of PCM includes a polyglutamine repeat, which is deleted at the protein level in the *pcm<sup>3</sup>* mutant. These data suggest that protein motifs within the C-terminus of PCM, possibly including the polyglutamine repeat, are involved in the localisation of PCM to P-bodies.

We then examined the effects of these *pcm* mutations on P-bodies using the P-body marker dDcp1. In these *pcm* mutants, P-bodies are dramatically increased in size and number in a similar way to that observed in yeast cells mutant for Xrn1 (Figure 5, panels D-F). Quantitation of the size difference reveals that the particles are increased 2.7 times in *pcm<sup>5</sup>* mutants compared to controls (Figure 5, panel G). These results provide additional evidence that the testis granules marked by PCM and Dcp1 are equivalent to yeast P-bodies.

The increase in dDcp1 localisation to P-bodies could be due to an increase in dDcp1 expression or recruitment of dDcp1 from the cytoplasm to these RNA granules. To test this, we performed Western blotting experiments on hand-dissected testis from wild-type and *pcm* mutants using the dDcp1 antibody. These results showed that the amount of dDcp1 decreases in *pcm* mutants (Figure 5, panels H and I), supporting the notion that the increase in Dcp1 staining in P-bodies in *pcm* mutants is due to massive recruitment of this protein to P-bodies.

### Pacman foci increase in size and number upon heat stress

In yeast and human tissue culture cells, environmental stresses can result in increases in sizes and numbers of P-bodies. Stresses such as glucose deprivation, osmotic stress and UV irradiation increase the size and numbers of P-bodies in yeast cells [24]. Although heat stress has not been reported to lead to an increase in size or numbers of P-bodies in yeast [25], the heat stress used was relatively mild (37°C) so may not have been strong enough to elicit a P-body response [36]. In human DU145 cells, heat shock (44°C for 1 hour) can result in an increase in numbers of P-bodies [24].

If these testis granules are akin to human P-bodies, we would expect them to increase in size when the male flies are stressed. To test this prediction we subjected male *Drosophila* to heat stress. We used heat stress as it is easy to administer to live flies and has already been shown to affect male fertility without substantially compromising viability [37]. Our experiments show that, in testes dissected from males subjected to heat shock (37°C for 1 hour), the numbers and sizes of granules marked by PCM increases 4.1-fold (Figure 6,

panels A and B). In addition, granules marked by dDcp1 also increase between 1.3 and 2.1-fold in wild-type and mutant testis when males are heat-shocked (Figure 6, panels D - L). The response of these testis granules to heat stress is very similar to that seen in human tissue culture cells, confirming that these granules are likely to be P-bodies [24]. These results raise the possibility that P-bodies are important in the response of testis cells to stress and suggest that mRNA turnover and its relationship to translation is similar between *Drosophila* and humans.

## Discussion

### Pacman is located in particles akin to P-bodies in *Drosophila* testes

PCM is located in cytoplasmic particules in both spermatogonia and spermatocytes. What is the function of these particles? Data in this paper suggests that they are akin to P-bodies, which are thought to be involved in the degradation of RNAs. The evidence for this is that PCM (an exoribonuclease) is co-localised with the decapping activator Dcp1 and the helicase Me31b (a Dhh1 homologue). In addition, we have shown that PCM is physically associated with dDcp1. Dcp1 is often regarded as a marker for P-bodies [34]. In addition, these particles behave in the same way as yeast and human P-bodies in that they increase in size upon stress (Figure 6) and also in mutants where members of the 5' - 3' pathway is downregulated.

Although elegant work in yeast has shown that P-bodies accumulate partially degraded mRNAs, further work has shown that P-bodies can also store mRNAs. Therefore the function of P-bodies in yeast is not yet clear. However, our recent work examining PCM localisation of PCM in *Drosophila* egg chambers has shown the existence of two types of particle containing PCM, dDcp1 and dDcp2: one type in the nurse cell cytoplasm which responds to stress and depletion of RNA decay factors and the other type in the oocyte which is resistant to these factors [38]. Since the oocyte particles are also co-localised with known translational repressors such as Oskar, these appear to be storage bodies, rather than particles involved in mRNA degradation [38]. Furthermore, in *Drosophila* neurones, PCM is co-localised with translation repression factors such as dFMR1, Cup and Pumilio in neuronal granules [39], suggesting that it is also involved in translational repression in these tissues. Although we cannot rule out that the PCM particles we see in testis are storage granules, their response to stresses and depletion of mRNA factors, together with their small size, suggests to us that they are involved in RNA degradation.

The large size of spermatocytes has allowed us to analyse the internal structure of PCM-containing granules in a natural tissue in a way that has not previously been possible in yeast or human tissue culture cells. As shown in Figure 4, Me31b and dDcp1 are almost completely co-localised with each other in these particles, whereas PCM is partially co-localised with both. Note that this co-localisation has been seen using antibody staining of wild-type testis, showing that co-localisation is not an artifact arising from over-expression of fusion proteins. These results are similar to that seen in the cytoplasm of nurse cells in *Drosophila* egg chambers, where PCM is partially colocalised in particles with Me31b and dDcp1 [38, 39]. These data suggest a scenario where the helicase Me31b and the decapping activator dDcp1 bind mRNA together and then bring it to the exoribonuclease PCM. Further experiments using GFP- and YFP-tagged proteins in live testis imaging are in progress to understand the dynamic nature of these particles in these natural tissues.

### Pacman affects male fertility

Our data show that the exoribonuclease *pcm* is required for normal male fertility in *Drosophila melanogaster*. Examination of *Drosophila* males carrying hypomorphic mutations in *pcm* show that their testes are small and abnormal and that they produce

fewer offspring compared to controls. The expression levels of *pcm* are correlated with the number of offspring produced as *pcm*<sup>5</sup> males (which express undetectable levels of truncated protein) produce fewer offspring than *pcm*<sup>3</sup> males, (expressing higher levels of mutant protein). These data show for the first time, that an exonuclease is required for spermatogenesis.

How could an exonuclease, which is relatively non-specific in-vitro [11] affect spermatogenesis? PCM is expressed at the tip of the testis, in the spermatogonia, showing that PCM is required in these mitotically dividing cells. In these cells, it is possible that *pcm* perturbs TGF-beta signalling as the mutant testes strongly resemble those mutant in the TGF-beta ligand *Punt* [40]. Indeed, in complementary experiments, we have shown that *pcm* genetically interacts with *puckered*, a member of the JNK signalling pathway that can regulate TGF-beta signalling [41]. TGFbeta signalling is required for maintenance of the germline stem cells: lack of the TGF-ligand *Glass bottom boat* (*Gbb*) results in a reduction of stem cells with the concomitant reduction of mitotically dividing spermatogonia. In spermatocytes, PCM may disrupt differentiation of spermatocytes by the inappropriate upregulation of target RNAs. Since *pcm* is highly conserved, and the spermatogenesis process has similarities between *Drosophila* and humans, it is also likely that human *Xrn1* affects human spermatogenesis. Our further work to elucidate the targets of PCM in *Drosophila* spermatogenesis will therefore promote understanding of gene regulation in human germ cells.

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## Figure Legends.

### Figure 1.

(A) Diagram showing the genomic structure of *pcm*. Exons (■), introns (—), the “domino” repeat (QQEAQ), the polyglutamine repeat (polyQ) and the poly-adenylation site is shown. The insertion site of the P-element used to generate deletions in the *pcm* gene is given, as is the downstream gene CG12202 (Nat1). The *pcm* alleles described in this paper are given below: gaps represent deletions at the genomic DNA level. (B) Western blotting of *pcm* hemizygous mutant male testes showing that the truncated *pcm* proteins are not detectable (*pcm*<sup>5</sup>) or are poorly expressed (*pcm*<sup>3</sup>). An actin loading control is shown below. (C) Histogram of normalized expression levels relative to loading controls. Means and standard errors are given.

### Figure 2.

Effects of *pcm* on spermatogenesis. (A) *pcm* mutant males produce fewer offspring than controls. Means and standard errors are given. (B) *pcm* mutant males produce fewer mature sperm than controls. Seminal vesicles were dissected from 3-day old virgin males at 25°C and the mature sperm counted. Means and standard errors are given. (C), (D) *pcm* mutant testes are shorter (C) and thinner (D) than controls. Means and standard errors are given. (E-G) *pcm* mutant testes are smaller and “weedier” than controls. (E), (F), and (G) represent testis dissected from 2-day old wild-type, *pcm*<sup>3</sup>, and *pcm*<sup>5</sup> males, respectively.

### Figure 3.

Localization of PCM in testis cells. (A) PCM antibody staining shows that PCM is highly expressed at the tip of the testis. (B) PCM is expressed in cytoplasmic foci in spermatogonia. Scale bar: 10 µm. (C) GFP-PCM is expressed in cytoplasmic foci in spermatogonia from transgenic males. (D-F) GFP-PCM expressed in spermatocytes in transgenic males and (D) co-localizes with PCM detected using PCM antibody (E). (F) Merged image. Scale bar: 10 µm.

### Figure 4.

PCM is partially co-localized with the mRNA decapping enzyme dDcp1 and the helicase Me31B in P-bodies. (A, B) dDcp1 antibody staining (A) and YFP-dDcp1 expressed in testis cells in transgenic males (B) showing that dDcp1 is expressed in cytoplasmic foci in testis cells. Scale bars: 10 µm. (C-E) YFP-dDcp1 expressed in testis cells in transgenic males (C) is partially co-localized with PCM detected using PCM antibody (D). (E) Merged image. Scale bar: 2 µm. (F-H) The helicase Me31B (F) is partially co-localized with PCM (G) in cytoplasmic particles in testis cells. (H) Merged image. Scale bar: 2 µm. (I-K) The helicase Me31B (I) is partially co-localized with dDcp1 (J) in cytoplasmic particles in testis cells. (K) Merged image. Scale bar: 2 µm. (L) PCM and dDcp1 proteins physically interact. Protein extracts were prepared from wild-type (control and wt lanes) or *pcm*<sup>5</sup> testes. These were used directly (input - labelled 1) or immunoprecipitated (labelled 2) with (wt and *pcm*<sup>5</sup>) or without (control) our anti-PCM antibody. No crosslinking procedures were used. Extracts were then electrophoresed on a non-denaturing gel and Western blotted using an anti-dDcp1 antibody. In the *pcm* mutant the amount of dDcp1 co-precipitated is less than in the wt as expected.

### Figure 5.

(A-C) PCM antibody staining shows that cytoplasmic particles are more diffuse in *pcm* mutants than controls. (A), (B), and (C) represent wild-type, *pcm*<sup>3</sup>, and *pcm*<sup>5</sup> testis, respectively. Images for *pcm* mutants (B, C) were taken at a higher power of confocal

laser since no signal is seen when a power equivalent to panel **(A)** was used. **(D-F)** dDcp1 antibody staining shows that cytoplasmic particles are larger in *pcm* mutants than control. **(D)**, **(E)**, and **(F)** represent wild-type, *pcm*<sup>3</sup>, and *pcm*<sup>5</sup> testis, respectively. **(G)** dDcp1 cytoplasmic particles are larger in *pcm* mutants than control. Means and standard errors are given. The observed differences for the size of the particles between the control and *pcm*<sup>3</sup> and control and *pcm*<sup>5</sup> were statistically significant in both cases (Mann-Whitney test,  $W=422.5$ ,  $P=0.0106$  and  $W=632.5$ ,  $P<0.0001$ , respectively). Scale bar: 5  $\mu\text{m}$ . **(H)** Western blotting of *pcm* hemizygous mutants (*pcm*<sup>3</sup>/Y and *pcm*<sup>5</sup>/Y) showing that dDcp1 is expressed at lower levels in mutants compared to controls. An actin loading control is shown below. **(I)** Histogram of normalized expression levels relative to loading controls. Means and standard errors are given.

### Figure 6.

Under stress conditions the cytoplasmic particles containing PCM and dDcp1 increase in size. **(A-C)** Particles containing PCM in wild-type spermatogonias increase in size upon stress. **(A)** Normal condition. **(B)** Stress condition. **(C)** PCM cytoplasmic particles increase in size under stress conditions. Means and standard errors are given. The observed difference between the normal and stress conditions was statistically significant (Mann-Whitney test,  $W=2.5$ ,  $P<0.0001$ ). **(D-L)** Particles containing dDcp1 in wild-type **(D-F)**, *pcm*<sup>3</sup> **(G-I)**, and *pcm*<sup>5</sup> **(J-L)** spermatogonias increase in size upon stress. **(D, G, and J)** Normal condition. **(E, H, and K)** Stress condition. Scale bar: 5  $\mu\text{m}$ . **(F, I, and L)** dDcp1 cytoplasmic particles in wild-type **(F)**, *pcm*<sup>3</sup> **(I)**, and *pcm*<sup>5</sup> **(L)** increase in size upon stress. Means and standard errors are given. The observed difference between the normal and stress conditions in wild-type, *pcm*<sup>3</sup>, and *pcm*<sup>5</sup> was statistically significant (wild-type, Mann-Whitney test,  $W=49$ ,  $P<0.0001$ ; *pcm*<sup>3</sup>, Mann-Whitney test,  $W=125.5$ ,  $P=0.0054$ ; *pcm*<sup>5</sup>, exact permutation test,  $T=262$ ,  $P=0.049$ ).

Figure 1

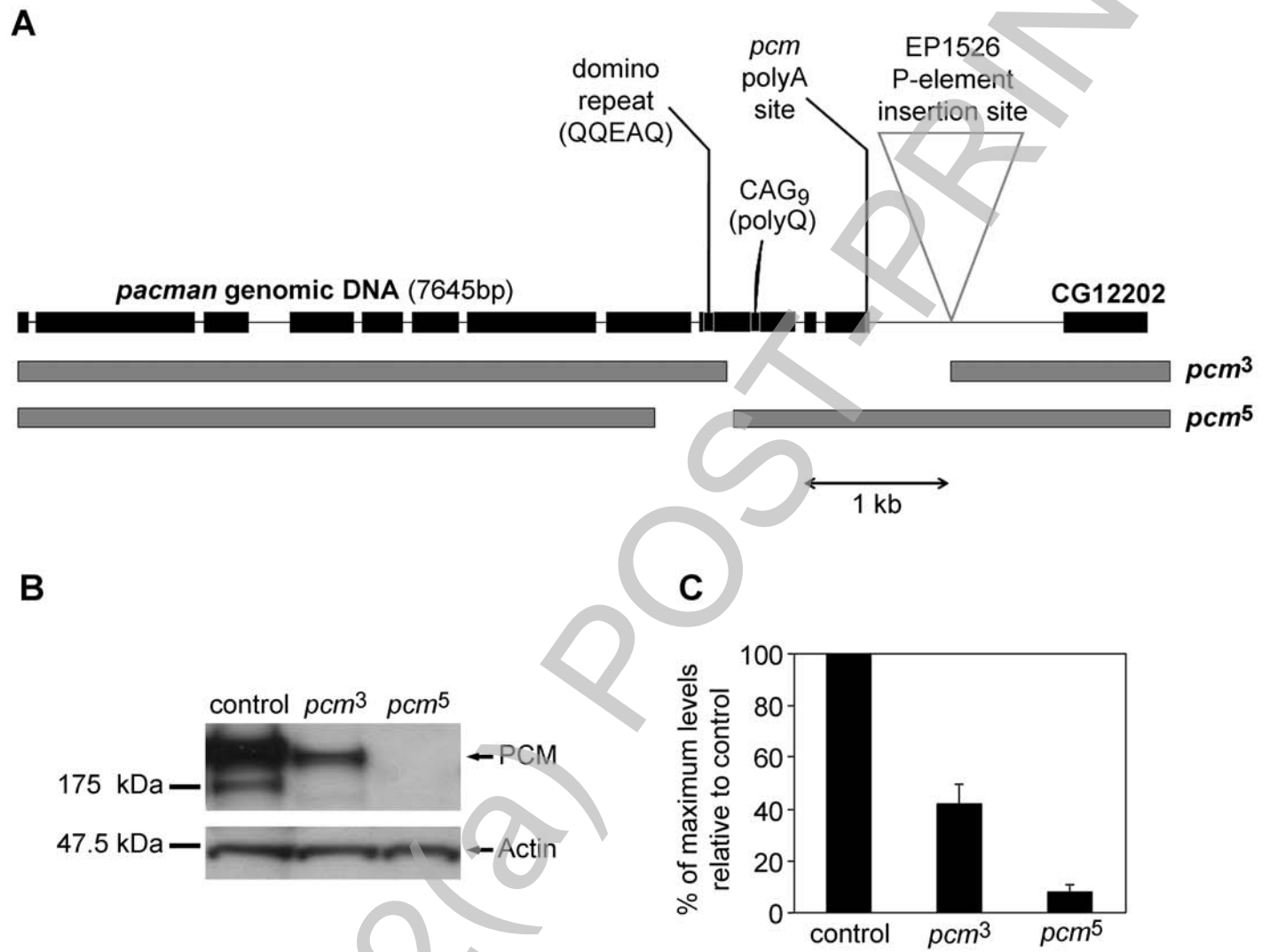
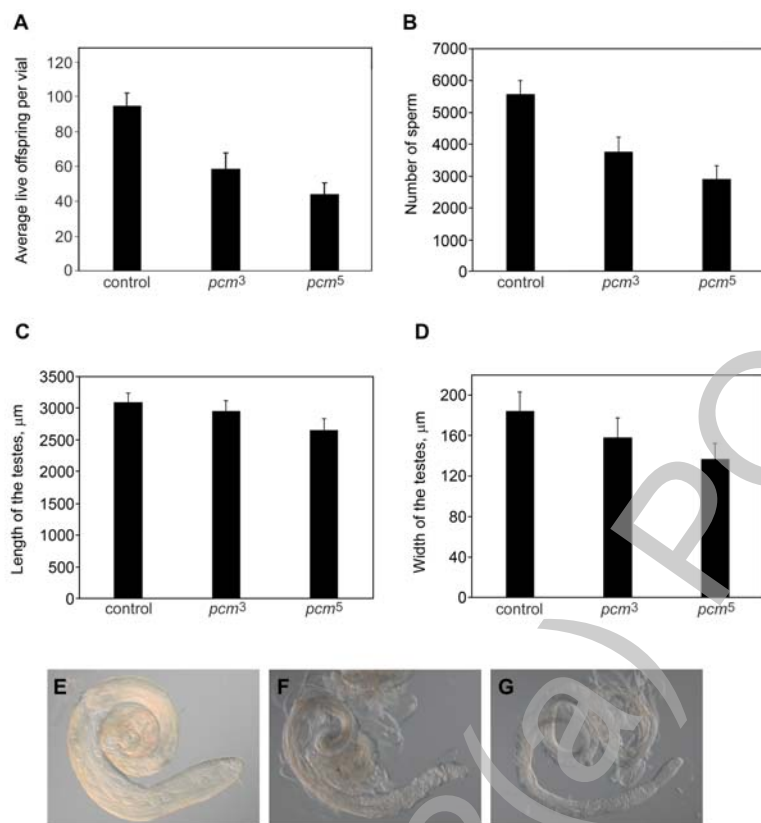
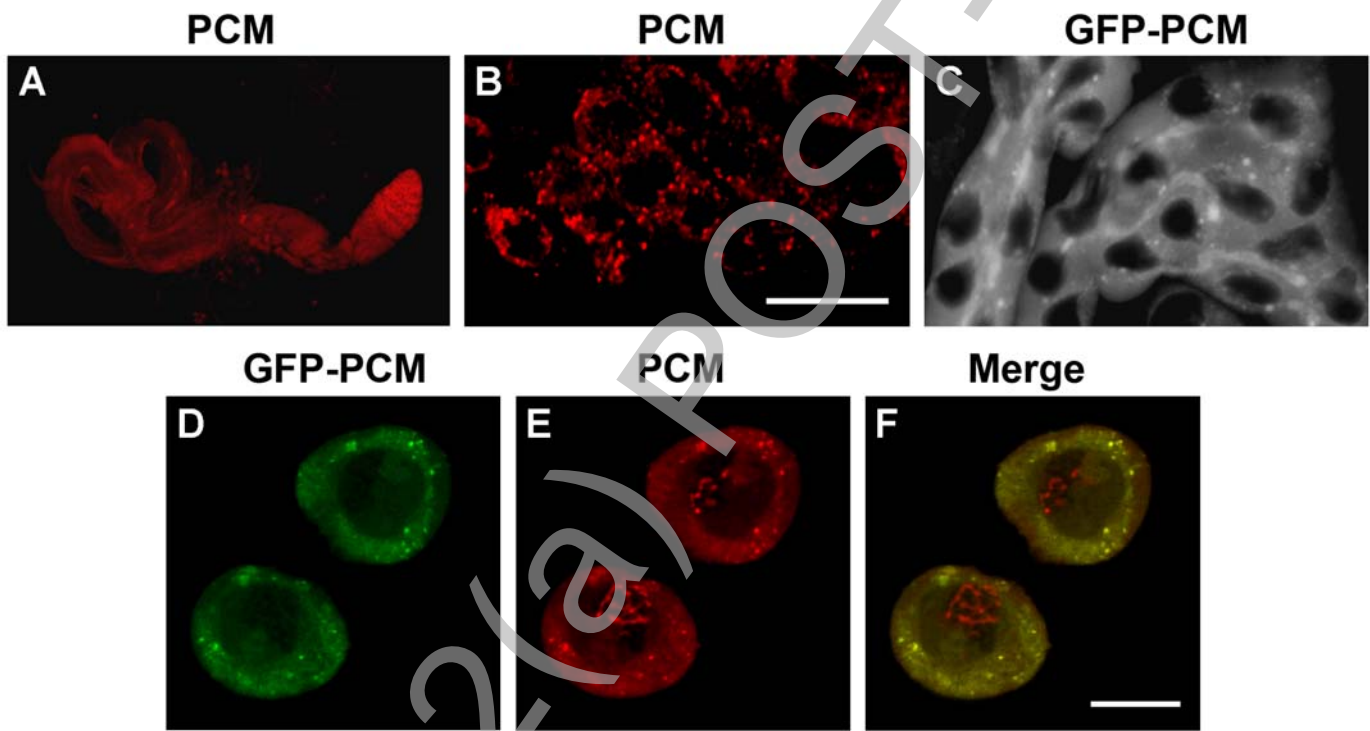


Figure 2

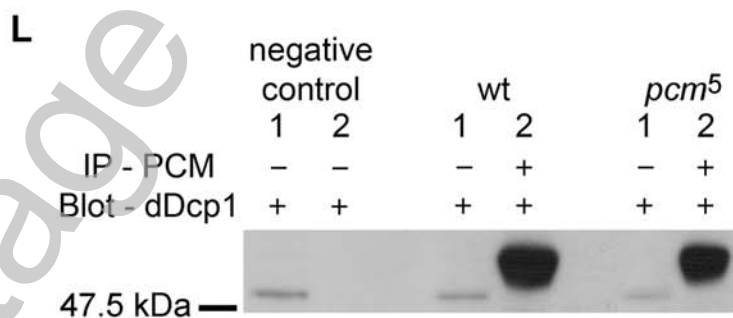
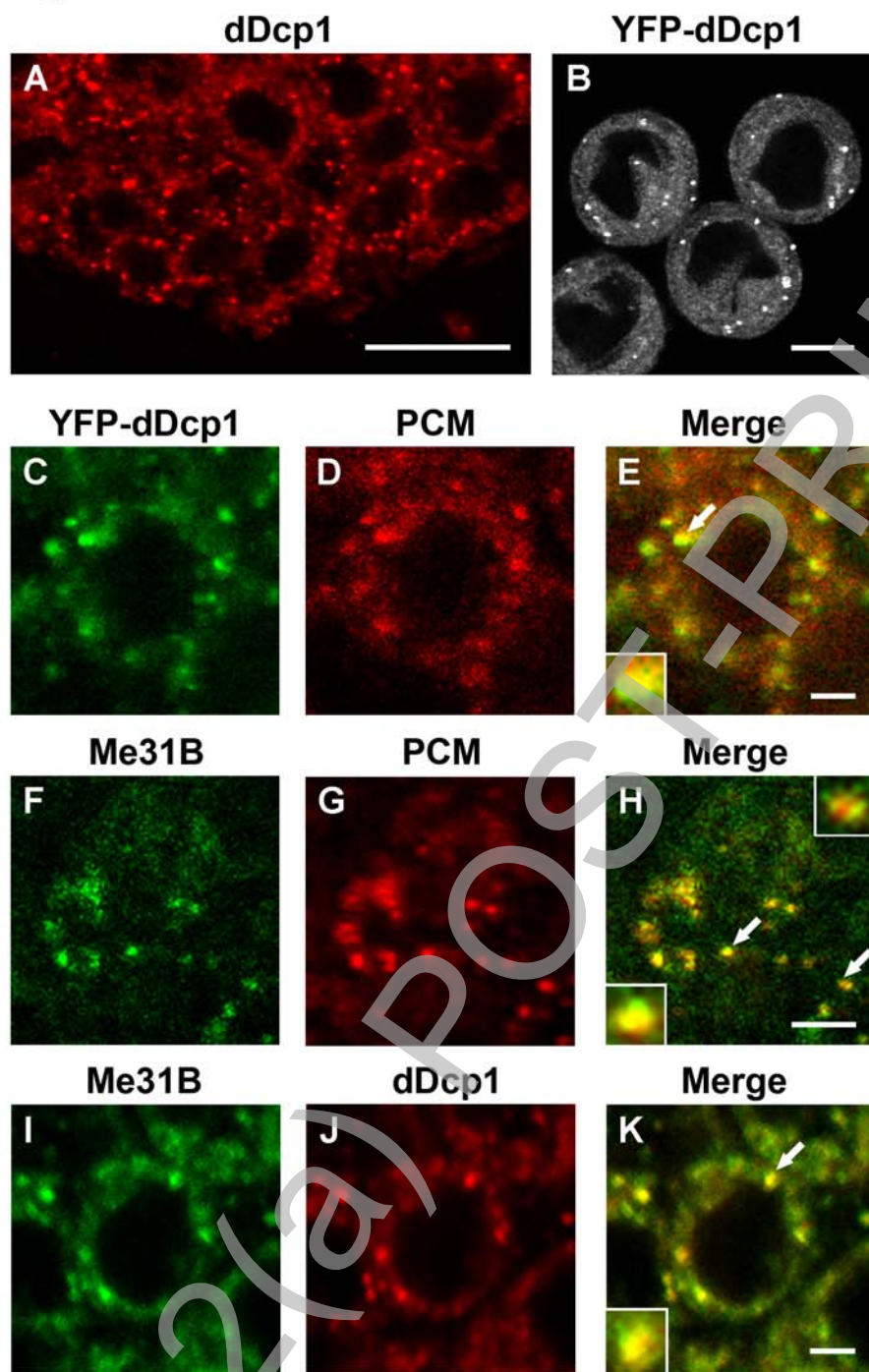


**Figure 3**



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**Figure 4**



**Figure 5**

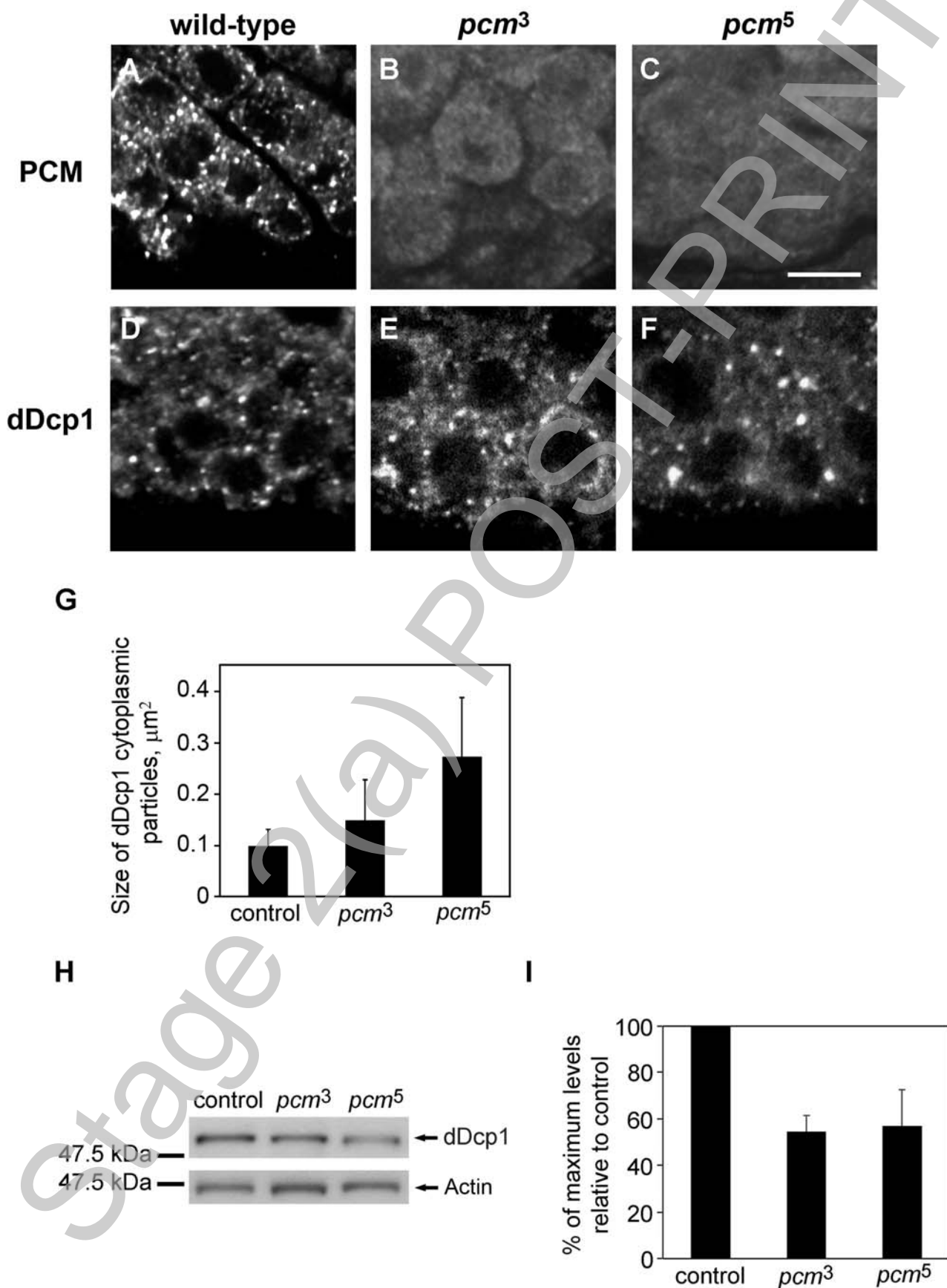
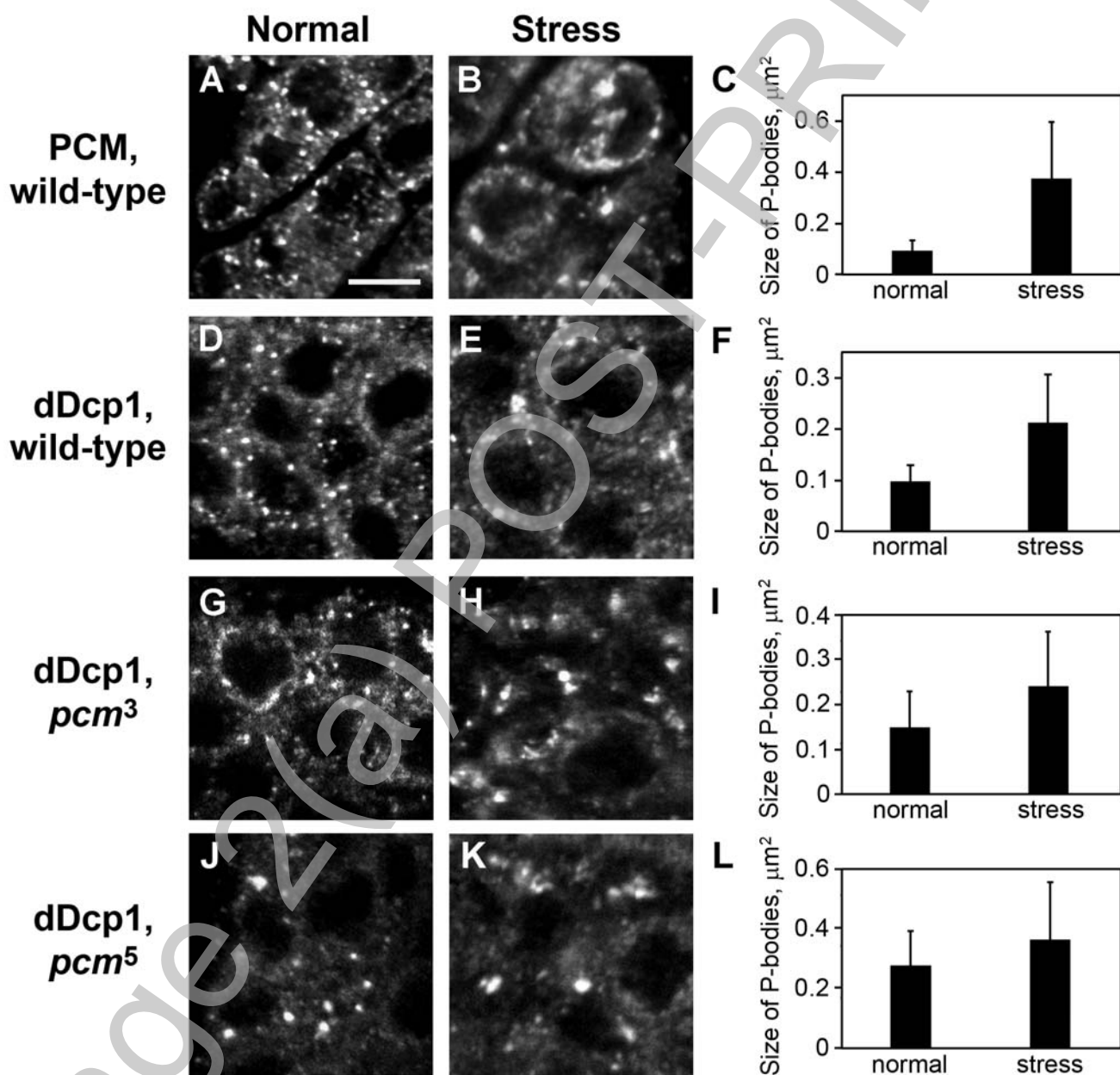


Figure 6



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