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giant nuclei is essential in the cell cycle transition from meiosis to mitosis

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SUMMARY

At the transition from meiosis to cleavage mitoses, *Drosophila* requires the cell cycle regulators encoded by the genes, *giant nuclei* (*gnu*), *plutonium* (*plu*) and *pan gu* (*png*). Embryos lacking Gnu protein undergo DNA replication and centrosome proliferation without chromosome condensation or mitotic segregation. We have identified the *gnu* gene encoding a novel phosphoprotein dephosphorylated by Protein phosphatase 1 at egg activation. Gnu is normally expressed in the nurse cells and oocyte of the ovary and is degraded during the embryonic cleavage mitoses. Ovarian death and sterility result from *gnu* gain of function. *gnu* function requires the activity of *pan gu* and *plu*.

Key words: *Drosophila melanogaster*, Mitosis, DNA replication, Fertilization, Oogenesis, Protein phosphatase 1.

INTRODUCTION

Natural developmental mechanisms ensure that oocytes and eggs arrest to await fertilization. These exhibit remarkable evolutionary flexibility, with different species using a variety of discrete arrest points, illustrating the diversity of regulatory mechanisms that have evolved to arrest the fundamental cell cycle oscillator (Sagata, 1996). The *Drosophila* oocyte is normally arrested by its chiasmate chromosomes at metaphase of the first meiotic division (Jang et al., 1995). Movement of the oocyte into the oviduct is accompanied by its hydration and activation (Heifetz et al., 2001) to complete both meiotic divisions resulting in three polar bodies and one female pronucleus that share a common cytoplasm. In unfertilised eggs, the four meiotic products arrest with condensed chromosomes.

At fertilization, Cdks and their activators are present in excess in the *Drosophila* embryo as a result of maternal provisioning. From mitotic cycle 8, global Cyclin A and B levels oscillate, generating fluctuations in Cdk1 activity (Edgar et al., 1994). However, prior to cycle 8 the global levels of Cyclin A and B appear not to oscillate and global Cdk1 levels and activity, as measured by histone H1 kinase levels and tyrosine phosphorylation status, are constant (Edgar et al., 1994). Recent evidence suggests that Cyclin degradation and Cdk activity oscillation are localised (Su et al., 1998; Huang and Raff, 1999), which may explain how syncytial nuclei are able to exit mitosis despite the presence of high Cyclin levels and Cdk1 activity in the rest of the embryo.

plu, *png* and *gnu* are three genes required maternally to inhibit DNA replication in the unfertilised egg and to couple S phase and mitosis in the subsequent embryonic cleavage cycles (Freeman et al., 1986; Freeman and Glover, 1987; Shamanski and Orr-Weaver, 1991; Axton et al., 1994; Elfring et al., 1997; Fenger et al., 2000). Regardless of embryonic genotype, oocytes, eggs and embryos derived from *plu*, *png* or *gnu* homozygous females will be referred to here as *plu*, *png* or *gnu* oocytes, eggs or embryos.

Pan gu and Plu co-immunoprecipitate from egg and embryo extracts suggesting they act in a complex. However, the level of Plu is reduced in null *png* mutants (Elfring et al., 1997) leaving open the possibility that Plu is a downstream effector of Pan gu. The levels of the mitotic Cyclins A and B and Cdk1 kinase activity are decreased in embryonic extracts mutant for *png*, *gnu* or *plu* (Fenger et al., 2000) providing a link between the giant nuclei phenotype with known cell cycle regulators. In addition, a genetic screen for *png* genetic interactors identified enhancement by *cyclin B*. Experimental restoration of Cyclin B levels in a *png* background was able to restore polar body chromosome condensation, though the zygotic nuclei eventually became polyploid (Lee et al., 2001). Thus Cyclin B is a critical, but probably not the sole, target of *png*, *plu* and *gnu* action. Here we describe the cloning of the *gnu* gene. We also describe an analysis of the *gnu* over-replication phenotype and investigate *gnu* function using epitope-tagged constructs.

MATERIALS AND METHODS

Drosophila stocks and libraries

Stocks (Lindsley and Zimm, 1992) were maintained at 25°C under

standard conditions (Roberts and Standen, 1998). The single extant *gnu* mutation [(Freeman et al., 1986) Tübingen stock *gnu*^{305}] was produced in a screen for female steriles by EMS mutagenesis of *ru th st kni*^{ri} *roe p*^{p} *e*^{s} *ca*. *gnu* complemented *Df(3L)fzD21* and *Df(3L)BrdR15* but was uncovered by *Df(3L)fzM21* and *Df(3L)D5rv5*. The phage library was constructed by C. Gonzalez in *Bam*HI-cleaved λDASH (Stratagene). The cosmids were in NotBamNot CosPer vector (Tamkun et al., 1992) and Lorist6 (Siden-Kiamos et al., 1990).

P-element induced male recombination

gnu was mapped by P-element induced male recombination (Chen et al., 1998) relative to *Trl*^{s2325} (a P-element insertion verified by inverse PCR). The *gnu* stock *ru gnu kni*^{ri} *th p*^{p} *e*^{s} */ TM3 Sb* has visible flanking markers *ru* and *kni[ri]*. The source of transposase was *Delta2-3 CyO*. Six independent recombinant chromosomes were recovered and all indicated that *gnu* is proximal relative to *Trl*^{s2325}.

Transgenes

A 3.5 kb *Xba*I fragment from phage clone 23.13.3 was transferred into the *Xba*I site of pCaSpeR4 (Sambrook et al., 1989). This comprised the complete ORF of CG5272, with 1.6 kb of 5′ and 0.9 kb 3′ sequence and the CG5258 (*NHP2*) coding region including the stop codon but lacking the 3′ UTR. This construct, inserted (Spradling, 1986) on the second chromosome (F1I) and an independent insertion (M2A) restored fertility to homozygous *gnu* females such that they produced fertile adult progeny. The premature stop codon and *Spe*I site were introduced by site-directed mutagenesis using the QuikChange™ (Stratagene) strategy with the primers CTGAGGCAGGAGGAAT-ACTAGTTGAAAAGTGCGCG and CGCGCACTTTTCAACTAG-TATTCCTCCTGCCTCAG. The mutated fragment was cloned into *Eco*RI/*Xba*I-cut pCaSpeR4. This construct inserted on the second chromosome (stock GS3A) and independent insertion (GS2B) did not rescue the sterility of homozygous *gnu* females. The eggs laid by such females failed to undergo any normal cleavage cycles and all developed giant nuclei.

Production of GFP-tagged *gnu* constructs

The 3.5 kb *Xba*I fragment in pBluescript SK was treated to remove the downstream CG5258 (*NHP2*) gene and destroy a vector *Bam*HI site, by *Pme*I/*Bam*HI digestion, end filling and re-ligation. A *gnu* 3′ *Bam*HI site was created by site-directed mutagenesis with the primers GCCAAGCAATTCTTCGGATCCTATATCCTGTAGG and CCTA-CAGGATATAGGATCCGAAGAATTGCTTGGC. Enhanced GFP (Cormack et al., 1996) was amplified using the restriction site-tagged primers CGGGATCCAAAGGAGAAGAACTTTTCACTG and CG-GGATCCTATTTGTATAGTTCATCCATGC and inserted into the new *gnu* 3′ *Bam*HI site. The entire insert was amplified by PCR using the restriction enzyme-tagged primers GCTCTAGAGCTCAGCTGTT-TCTTAGCC and GGAATTCAAGCATACTAGCGTGCCGC and the product was inserted into *Eco*RI/*Xba*I-digested pCaSpeR4 to create a genomic *gnu*-GFP construct. This construct, inserted on the second chromosome, restored fertility to homozygous *gnu* females (GG4c). Eggs laid by such females hatched and produced fertile adults. The rescue was complete since no giant nuclei were observed in unfertilised eggs or fertilised embryos from homozygous *gnu* females with the construct.

For Gnu-GFP mis-expression, a UASp *gnu*-GFP construct was produced by PCR using the restriction-tagged primers AAGGA-AAAAAGCGGCCGCATTATTTGTAAAATTACCG and GCTCT-AGAGGATCCTATTTGTATAGTTC and the genomic *gnu*-GFP construct as a template. The fragment was subcloned into *Not*I/*Xba*I-cut pSK. The fragment was excised and inserted into *Not*I/*Xba*I-cut UASp (Rørth, 1998). The inserts of all transformation constructs were sequenced in their entirety and no coding changes were found.

Embryo and ovary fixation, staining and microscopy

Protocols were from Sullivan et al. (Sullivan et al., 2000). Pictures were taken using an Eclipse 800 microscope (Nikon) with a MRC Radiance Plus laser scanning confocal system (Biorad) and LaserSharp software (Biorad) or a Nikon Optiphot attached to the BioRad MRC600 confocal microscope head. A Kahlman-averaging filter was used to reduce background. Our observations of GFP fluorescence are significant, since they were compared with identically-fixed oocytes and embryos not containing the *gnu-GFP* transgene and imaged with identical confocal settings.

DNA was stained with propidium iodide, primary antibodies used were YL1/2 rat IgG anti-alpha tubulin 1 μg/μl (Serotec Ltd) used at 1:500 dilution; T47 mouse monoclonal anti-lamin (Frasch et al., 1986), rabbit polyclonal against *Drosophila* PCNA antigen (Ng et al., 1990) 1:500; mouse monoclonal anti-bromodeoxyuridine (BrdU: Becton Dickinson). Secondary antibodies (Jackson) used were fluorescein (FITC)-conjugated AffiniPure F(ab′)$_2$ fragment donkey anti-rat IgG (H+L) minimal cross reaction diluted to 1:400, the equivalent FITC anti-rabbit was used for PCNA, FITC anti-mouse for lamin and BrdU. For Fig. 1D, embryos were treated with 0.5 mg/ml BrdU in Schneider's *Drosophila* medium for 5 minutes.

Protein extracts and immunoblots

Proteins were extracted in 50 mM Tris-HCl pH 6.8, 100 mM NaCl, 1 mM benzamidine-HCl, 1 mM phenylmethylsulphonyl fluoride (PMSF), 2 mM dithiothreitol (DTT), 1 mM Na_3VO_4, 50 mM NaF, 10 mM β-glycerophosphate on ice. An equal volume of 2× SDS loading buffer was added and the sample boiled for 5 minutes. The phosphorylated form (in the ovary) and the dephosphorylated form (in the embryo) of Gnu and of Gnu-GFP were detected in the absence of phosphatase inhibitors but the phosphorylated form was not stable in unboiled extracts without their use. The samples were centrifuged at 20,000 ***g***. Supernatants were separated on 10% 37.5:1 (acrylamide/methylenebisacrylamide), 0.1% SDS, pH 8.8 gels and transferred to PVDF by semi-dry electrophoresis. All blots were standardised by amount of material (embryos or ovaries) loaded, blots were checked for protein loading by Indian ink staining and subsequently re-blotted with anti-actin antibody as an internal loading control. Rabbit anti-Gnu-peptide antiserum Rb86 (Moravian Biotechnology) was preabsorbed on fixed 5- to 24-hour embryos and used at 1:2000 dilution, mouse anti-GFP monoclonal antibody (Zymed) was used at a 1:750 dilution and detected using a peroxidase-conjugated secondary (Vector) at a 1:30,000 dilution and Supersignal substrate (Pierce).

RESULTS

gnu eggs and embryos develop giant polyploid nuclei in which DNA replication is uncoupled from nuclear division

DNA replication in *gnu* eggs and embryos might be continuous or cyclic, with gaps in which there is no replication. To determine between these possibilities, we examined the distribution of the DNA polymerase-δ processivity factor, PCNA (Yamaguchi et al., 1991) and nuclear lamins in *gnu* embryos. In *gnu* embryos, the majority of giant nuclei stained for PCNA indicating that they were in S phase. However PCNA was excluded from a number of nuclei (Fig. 1A,B) even in the presence of a nucleus containing PCNA within the same embryo, suggesting that giant nuclei exit S phase and that the nuclei in a single *gnu* embryo do not always cycle in synchrony. The majority of the nuclei were surrounded by an intact lamina but occasionally, giant nuclei were observed in which the nuclear lamins had dissociated (data not shown).

When BrdU incorporation was used to detect DNA

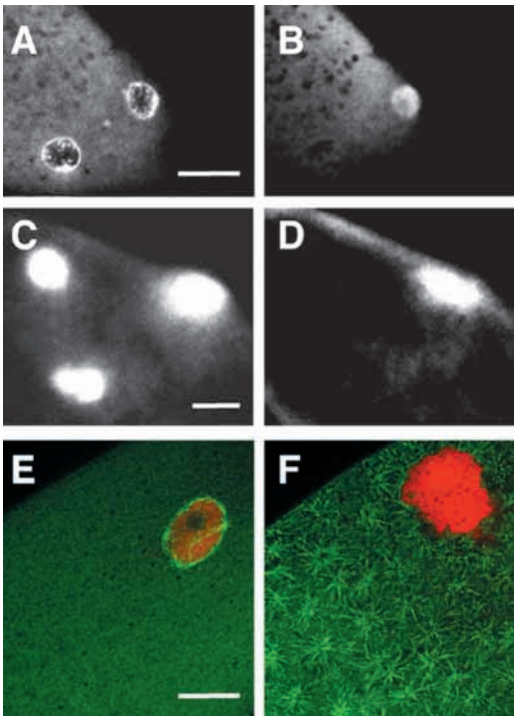

Fig. 1. DNA replication in *gnu* embryos. (A-D) Fluorescently immunostained eggs and embryos from *gnu* homozygous mothers. A nuclear lamina surrounded each of the giant nuclei in one embryo (A) but in this same embryo, only one nucleus stained for *Drosophila* PCNA (B). In another embryo, (C) DNA staining revealed three giant nuclei and, (D) following 5 minutes incubation, only one had incorporated BrdU. This indicated that not all of the nuclei are in S phase. (E,F) A 5 μm confocal section of an unfertilised egg (E) and embryo (F) stained for β-tubulin in green and DNA in red. Microtubule asters were initiated in the fertilised embryo from duplicating centrosomes, but were not present in the unfertilised egg. Scale bars: 50 μm (A-D), 25 μm (E,F) .

replication in *gnu* embryos, some, but not all, of the giant nuclei incorporated BrdU (Fig. 1C,D). Taken together, the results from these cell cycle markers suggest that some nuclei were in S phase whilst others in the same embryo were not and that DNA replication in *gnu* embryos is cyclic or of limited duration.

It was previously reported that, in *gnu* eggs and embryos, the nuclei neither condense chromosomes nor divide but the centrosomes replicate and organise asters apparently normally (Freeman and Glover, 1987). We examined microtubule asters in *gnu* eggs and embryos by staining with an antibody against tubulin. We found that asters were indeed initiated in *gnu* embryos in a regular array throughout the embryos, but that, in unfertilised eggs, the tubulin coated the giant nuclei and no asters were observed (Fig. 1E,F).

Gnu is a small novel protein

gnu lies between the distal breakpoint of *Df(3L)fzD21* at 70E5-6 and the distal breakpoint of *Df(3L)BrdR15* at 71A1-2. Microdissected clones of polytene chromosome DNA (Saunders, 1990) from the region were used as starting points to construct a genomic walk. By sequencing the ends of the inserts of phage and cosmid clones, we anchored the walk to the sequence of the *Drosophila* genome (Adams et al., 2000). *gnu* was mapped proximal to *Trl* by P-element-induced male recombination, placing *gnu* within a region of 131 kb and 10 predicted genes between *Trl* and the distal breakpoint of *Df(3L)BrdR15*. Sequencing genes from the *gnu* chromosome in this region revealed a C to T mutation in gene CG5272 resulting in a premature stop codon (Fig. 2). This mutation was not present on other lines (*fs(3)131-19* and *fs(3)135-17*, Tübingen stock centre) made in the same mutagenesis screen as *gnu* (data not shown).

In transgenic *Drosophila*, a 3.5 kb *Xba*I fragment from a phage containing CG5272, complemented the female sterility of the *gnu* mutation, however, transformants containing the same construct, except for a premature stop codon introduced into CG5272 by site-directed mutagenesis, did not rescue the *gnu* mutation. Therefore *gnu* is CG5272.

cDNAs GM10421 and LD12084, corresponding to ESTs in the BDGP database (http://www.fruitfly.org) that matched CG5272 were sequenced, confirming that *gnu* is a small gene with a single intron encoding a 240 amino acid protein with a predicted molecular mass of 27 kDa (Fig. 2). The premature stop codon in *gnu* mutants would produce a truncated protein lacking the C-terminal 94 residues. The deduced Gnu sequence was used to search the protein databases. No close matches were found, therefore Gnu is a novel protein.

Gnu is specific for early development

Rabbit anti-Gnu antiserum Rb86, raised against a synthetic peptide comprising aa117-131 is specific for Gnu and for Gnu-GFP but does not recognise a truncated product of the *gnu* mutant (Fig. 3A). The expression profile of a functional Gnu-GFP fusion protein under the control of the *gnu* promoter was examined by immunoblotting and detection with a monoclonal antibody against GFP. Gnu-GFP was expressed in ovaries and 0- to 3-hour embryos (Fig. 3A,B) and in unfertilised eggs, but not in larval tissues or in adult testes (not shown). The epitope-tagged protein had very similar expression to native Gnu detected with an anti-peptide antiserum, but had a somewhat longer half-life in cleavage embryos. We did not detect Gnu in embryos more than 1 hour after egg deposition (Fig. 3B), in larvae or in adult testes (not shown). The mobility of Gnu and of Gnu-GFP from ovaries was slower than from unfertilised eggs or embryos suggesting Gnu is post-translationally modified. The mobility of the embryonic isoform matched the predicted size of the fusion protein (54 kDa). GFP mobility in extracts from ovaries and embryos from a *ubiquitin*-driven GFP line were identical (data not shown), therefore it is only the Gnu moiety of Gnu-GFP that is modified.

Gnu is dephosphorylated upon egg activation

In ovary extracts with phosphatase inhibitors, the slow moving form of Gnu-GFP was observed (Fig. 3A,C). If phosphatase inhibitors were omitted from the ovary extraction, the amount of slow moving form was reduced in favour of the fast moving form with the same mobility as Gnu-GFP from embryos (Fig. 3C). To ascertain which protein phosphatases (PPs) are involved, specific inhibitors of serine/threonine protein phosphatases were tested for their ability to stabilise the slow moving form (Fig. 3C). Okadaic acid (OA) at low concentration (1 nM), sufficient to inhibit PP2A, did not

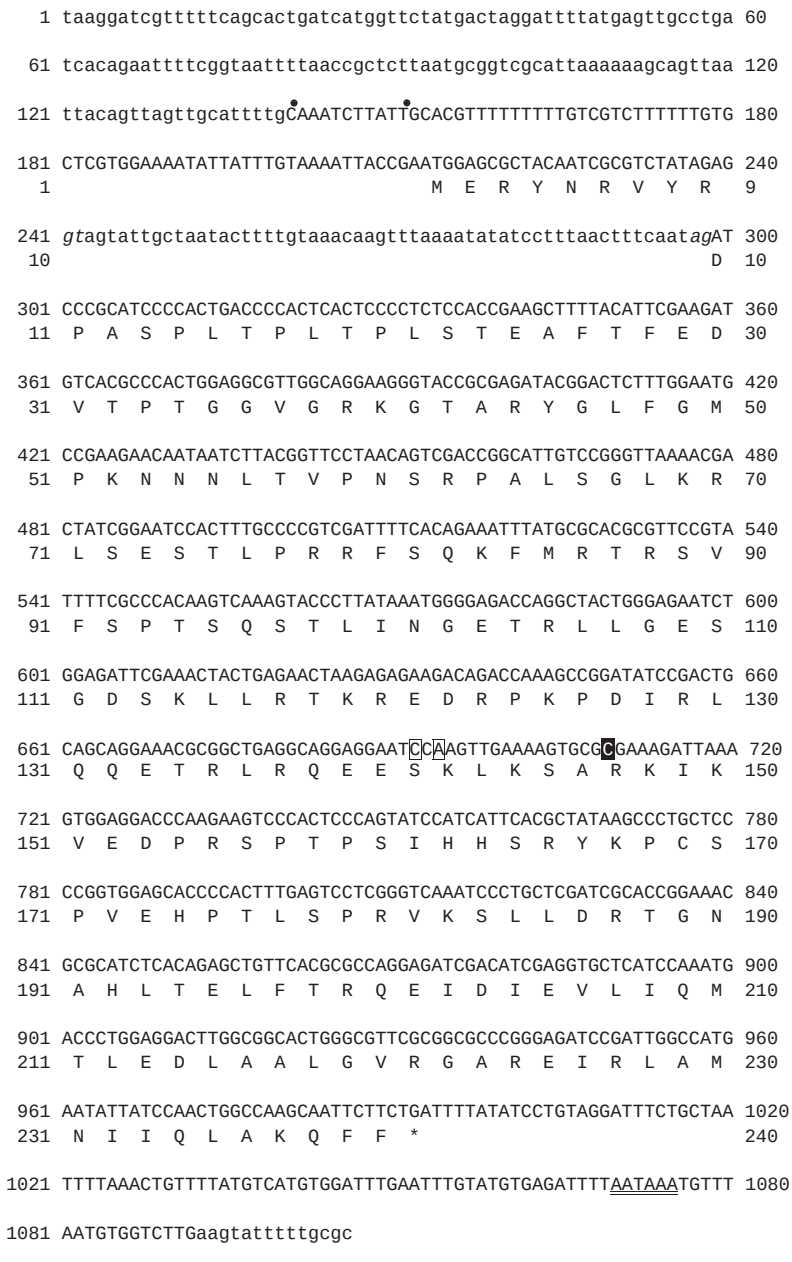

Fig. 2. *gnu* DNA and deduced protein sequence (EMBL Accession Number AJ557828). The genomic sequence of *gnu* from an isogenic *y; cn bw sp* stock (Adams et al., 2000) is shown with the exons in upper case and the predicted amino acid sequence shown below the DNA sequence. The consensus splice sites are in italic and the polyadenylation signal is doubly underlined. The site of the C to T mutation on the *gnu* chromosome (base 709) is indicated by a solid black box. The ESTs GM10421 and LD12084 begin at bases 141 and 151 respectively (denoted by •). On sequencing *gnu* from OregonR and from our genomic rescue construct the following polymorphisms were found: 91 T→C and 113 G→A, 165 extra T in our genomic rescue construct (numbers refer to bases with *y; cn bw sp* version first and polymorphism second). All of these polymorphisms are in untranslated regions except 324 C→A in OregonR which is a silent mutation in the coding sequence. Overall there is no polymorphism in the deduced protein sequence. The residues altered in the production of *gnuSTOP* (boxed), were 692 C→A and 694 A→T, resulting in a premature TAG stop codon in place of the lysine at 142 and a *Spe*I site (ACTAGT).

Gnu accumulates in oocytes of stage 11 egg chambers and is cytoplasmic in eggs and embryos

In ovaries, Gnu-GFP was first observed in fixed oocytes of stage 11 egg chambers (Fig. 4A). In subsequent stages it accumulated in the oocyte but was not observed in nurse cells (Fig. 4B). In eggs, Gnu-GFP was cytoplasmic and showed no association with the replicatively inactive polar body chromosomes (Fig. 4C,D). In syncytial embryos Gnu-GFP was again cytoplasmic at all stages of the cell cycle. Although the nuclear envelope stains somewhat more distinctly, Gnu is neither strongly localised within, nor excluded from zygotic nuclei (Fig. 4E,F).

Gnu post-translational modifications are not dependent on *pgn*

Gnu-GFP mobility in ovaries and eggs in both null and weak *png* backgrounds was indistinguishable from wild type (Fig. 3E). Therefore, Pan gu is neither the kinase that phosphorylates Gnu nor part of a pathway leading to Gnu dephosphorylation. Even if Pan gu does not influence Gnu modification, it might regulate Gnu localisation. To test this possibility we examined Gnu-GFP localisation in a *png* background. We found that Gnu was cytoplasmic in *png* embryos and it was excluded from the giant nuclei (Fig. 4G-I).

Gnu mis-expression in the ovarian germline results in sterility

We mis-expressed Gnu-GFP in *Drosophila* ovaries using the UAS-GAL4 system (Rørth, 1998; Brand and Perrimon, 1993). Females containing the *maternal alpha4tubulin>GAL4:VP16* driver and *UASp gnu-GFP* (see Materials and Methods) were sterile and did not lay eggs. Staining of their ovaries revealed Gnu-GFP was expressed from stage 5 onwards (Fig. 5A). Egg chambers up to stages 8-10 had wild-type morphology.

stabilise slow moving Gnu-GFP, however OA at a higher concentration (50 nM), sufficient to inhibit PP1 (Mackintosh and Mackintosh, 1994), stabilised slow moving Gnu-GFP. I-2Dm, a specific inhibitor of PP1 (Bennett et al., 1999), also stabilised slow moving Gnu-GFP. We conclude that PP1 can dephosphorylate Gnu in ovary extracts.

To determine the developmental time-point at which Gnu dephosphorylation occurs, we crossed the genomic *gnu-GFP* transgene into mutant backgrounds that cause the oocyte to arrest development during meiosis [*cortex* and *grauzon*e (Page and Orr-Weaver, 1996)], or immediately following meiosis but prior to the first zygotic mitosis [*deadhead* (Pellicena-Palle et al., 1997)]. Gnu-GFP mobility in ovaries and eggs in these mutant backgrounds was indistinguishable from wild-type (Fig. 3D) indicating that Gnu is dephosphorylated before meiotic arrest induced by *cortex* and *grauzone*, most likely at egg activation.

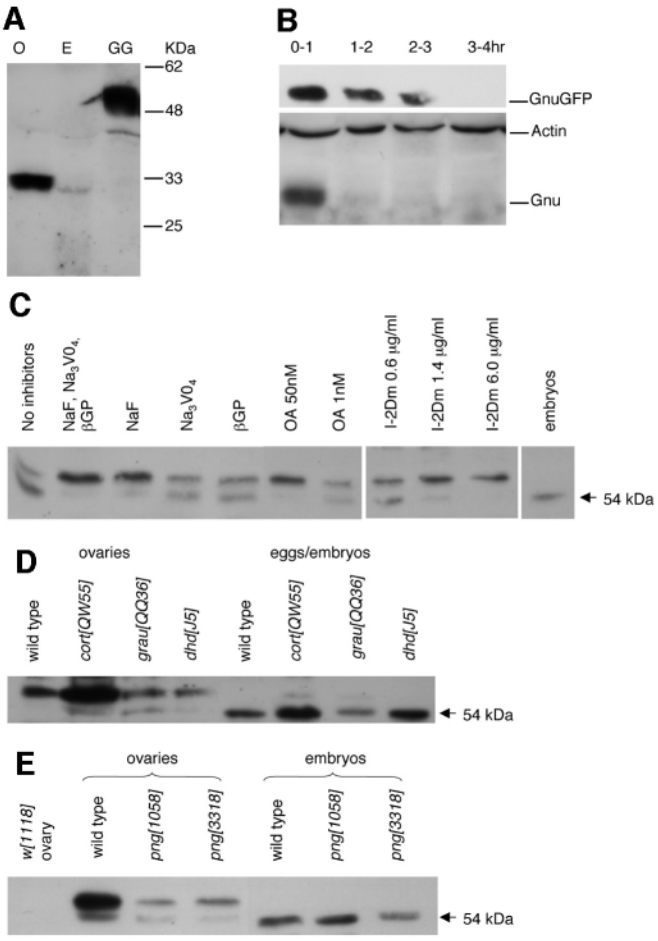

Fig. 3. Developmental expression and post-translational modification. (A) Immunoblot with anti-peptide antiserum detected native Gnu protein in wild type (*w[1118]*) *Drosophila* ovaries (O) and 0-3 hour embryos (E) and detected functional Gnu-GFP fusion protein in ovaries from stock GG4c: homozygous *gnu*, rescued by a homozygous insertion of the genomic *gnu-GFP* construct (GG). (B) Expression in staged 0- to 4-hour embryos of Gnu (anti-Gnu) in *w[1118]* embryos (loading control: anti-actin), Gnu-GFP (anti-GFP) in GG4c embryos. (C-E) Functional fusion protein detected with anti-GFP antibody. (C) Proteins extracted from GG4c ovaries in the presence of the protein phosphatase inhibitors fluoride (NaF), orthovanadate (Na_3VO_4), β-glycerophosphate (βGP), okadaic acid (OA) and Inhibitor-2 (I-2Dm). (D) Extracts of ovaries, eggs and embryos from flies containing a single insertion of the genomic *gnu-GFP* construct in *cortex* (*cort*), *grauzone* (*grau*) and *deadhead* (*dhd*) mutant backgrounds or wild-type control homozygous for the genomic *gnu-GFP* construct. (E) Extracts of ovaries and embryos from flies containing a single heterozygous insertion of the genomic *gnu-GFP* construct in homozygous *png* backgrounds. png^{1058} and png^{3318} are null and weak alleles respectively. The wild-type control is from flies homozygous for the genomic *gnu-GFP* construct.

However subsequent stages were characterised by large amounts of irregularly localised, often fragmented, chromatin resulting from the degeneration of nurse cell nuclei. No stage 14 egg chambers could be distinguished. Suprisingly, given that Gnu-GFP, expressed from its own promoter, was unlocalised in embryos, mis-expressed Gnu-GFP was exclusively nuclear in nurse cells (Fig. 5D-F).

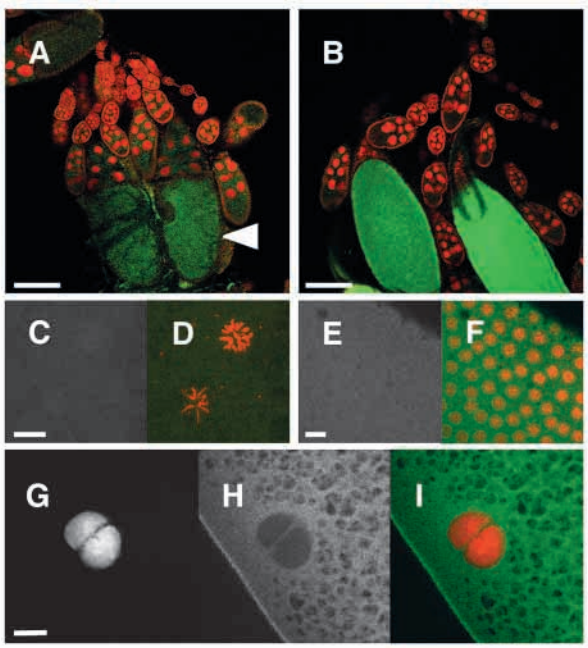

Fig. 4. Gnu localization in ovaries and embryos. Confocal sections of ovaries from *gnu* females rescued by the homozygous genomic *gnu-GFP* construct (stock GG4c). (A) Gnu-GFP fluorescence (green; DNA stained red) was first observed in oocytes of stage 11 egg chambers. (B) In subsequent stages, the fusion protein accumulated in the oocyte but was not observed in nurse cells. (C) Gnu-GFP fluorescence was not found to be specifically associated with the polar bodies (D). (E-F) Syncytial embryo in interphase of cycle 10. The Gnu-GFP fluorescence was cytoplasmic (E) and not excluded from nuclei (F). (G-I) Confocal sections of a giant nucleus from flies homozygous for png^{3318} and also containing a single heterozygous copy of the genomic *gnu-GFP* construct. (G) DNA, (H) Gnu-GFP fluorescence, (I) merged image with DNA in red and Gnu-GFP fluorescence in green. Scale bars: 100 µm (A,B), 10 µm (C-F), 20 µm (G-I).

Control females containing the same driver and a *UASp GFP* construct had wild-type ovarian morphology and were fertile (Fig. 5B). GFP was present throughout their nurse cells, though the nuclei had slightly higher levels than the cytoplasm (Fig. 5B). We concluded that GFP does not show particular affinity for nurse cell nuclei or impede oogenesis, therefore the nuclear accumulation and sterility arising from Gnu-GFP mis-expression resulted from the Gnu moiety.

We compared the mobility of ectopic Gnu-GFP from ovaries to that of Gnu-GFP from ovaries expressing the genomic construct. Mis-expressed Gnu-GFP from ovaries was identical to the dephosphorylated embryonic form (Fig. 5G). Thus nurse cells do not phosphorylate Gnu, suggesting that the Gnu kinase activity is restricted to the oocyte.

To test whether the sterility associated with Gnu mis-expression was a consequence of Gnu alone, we mis-expressed Gnu in ovaries in a *png* mutant background. Females homozygous for png^{1058} and containing the *maternal alpha4tubulin>GAL4:VP16* and *UASp gnu-GFP* constructs laid eggs (Table 1). Staining of their ovaries revealed they were morphologically normal with no abnormal egg chambers or fragmented DNA (Fig. 5C). The egg laying rates for such

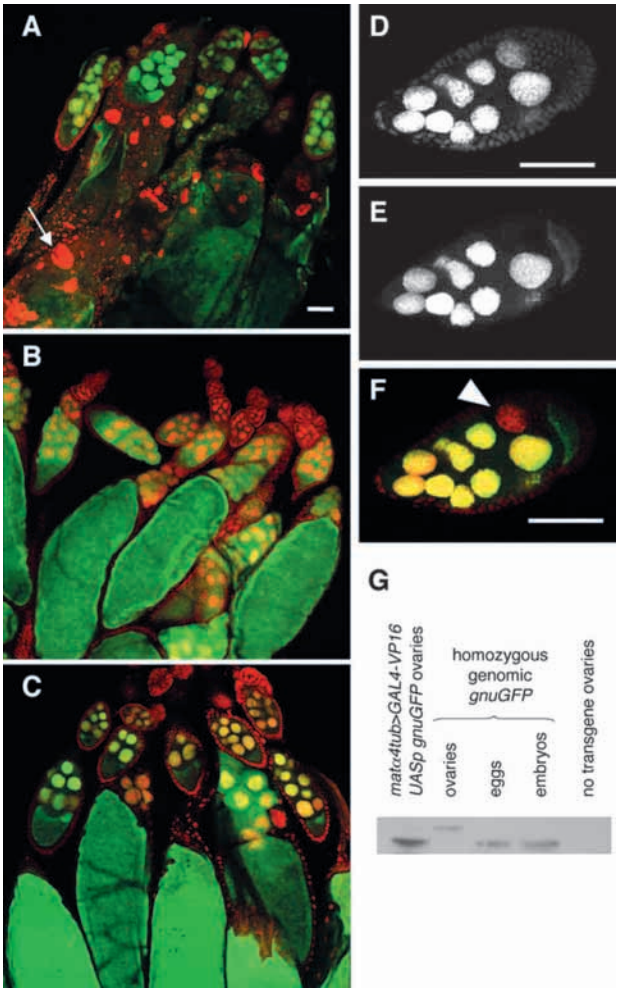

Fig. 5. Gnu mis-expression in ovaries results in sterility. Confocal sections of ovaries and egg chambers from females containing (A,D-F) the *maternal α4 tubulin>GAL4:VP16* and *UASp gnu-GFP*, (B) *maternal α4 tubulin>GAL4:VP16* with *UASp EGFP* constructs in a wild-type background, (C) *maternal α4 tubulin> GAL4:VP16* with *UASp gnu-GFP* in a homozygous png^{1058} null background. (A) Gnu-GFP mis-expressed in ovaries is nuclear and disrupts oogenesis with inappropriate nurse cell degeneration and fragmented chromatin (arrow). (B) Ectopic GFP is cytoplasmic and nuclear and does not disrupt ovary morphology. (C) Ectopic Gnu-GFP is largely nuclear but oogenesis is not disrupted and ovarian morphology is normal. DNA is red and GFP fluorescence, green. (D-F) A single stage 8 egg chamber. (D) Propidium iodide-stained DNA, (E) GFP fluorescence, (F) merged image of D and E, with DNA in red and Gnu-GFP fluorescence in green. Not all nurse cell nuclei contain Gnu-GFP (arrowhead). Scale bars: 50 μm. (G) Anti-GFP blot shows that Gnu-GFP expressed from the GAL4-UAS system is unmodified, in contrast with that expressed from the genomic *gnu-GFP* construct.

females were similar to wild type (Table 1) indicating that the restoration of ovarian function was complete. The eggs did not hatch but, when stained for chromatin, exhibited a giant nuclei phenotype identical to that in Fig. 4G-I, typical of *png* embryos. The earliest mis-expression and amount of Gnu-GFP fluorescence in a *png* background was the same as in a wild-type background indicating that the restoration of ovary

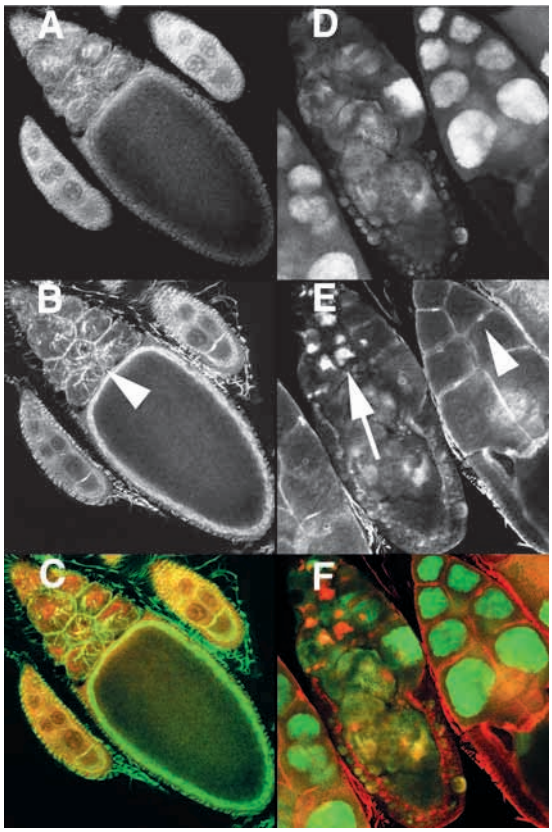

Fig. 6. Abnormal actin reorganisation in ovaries mis-expressing Gnu. (A) Propidium-stained DNA, (B) FITC-phalloidin stain for F-actin. (C) Merged image of A and B. DNA red, F-actin green. (D) Gnu-GFP in nurse cell nuclei. (E) Rhodamine-phalloidin stain for F-actin. (F) Merged image of D and E Gnu-GFP green, rhodamine-phalloidin red. Gnu-GFP mis-expression was compatible with some aspects of F-actin organisation, such as ring canals (E arrowhead, F). At the equivalent of wild-type stage 10, the actin cytoskeleton aggregated (E arrow) and did not develop the contractile meshwork (B arrowhead, C) that in the wild type ovary dumps nurse cell cytoplasm into the oocyte and retains nurse cell nuclei in oogenic stages 10B and 11. Confocal sections were taken of egg chambers from females containing the *maternal α4 tubulin>GAL4:VP16* (A-C) and *UASp gnu-GFP* (D-F) constructs in wild-type background.

function is not caused by an effect of *png* on Gnu-GFP mis-expression levels or timing. The effect of a homozygous null *plu* mutation in combination with Gnu-GFP mis-expression was indistinguishable from that of the null *png* mutation (Table 1).

Since the Gnu gain-of-function phenotype resembles that of Profilin mutants (Cooley et al., 1992) we examined the actin cytoskeleton of egg chambers (Fig. 6). Defects were first seen at stage 10, when nurse cells mis-expressing Gnu failed to assemble the actin meshwork and did not dump their cytoplasmic contents into the oocyte. We do not know whether the disruption of actin reorganisation is the primary consequence of excess Gnu, or whether premature egg chamber death results in the dramatically abnormal aggregates of F-actin. What is clear from our epistasis experiments is that this ‘dump-less’ phenotype is specific, in that it also requires Pan gu and Plu.

Table 1. Epistasis of *gnu* with *pgn* and *plu*

Genotype	Eggs/fly/day		Eggs		Phenotype	% surviving females		*n*
	Mean	s.d.	Hydrated	Hatched		4 days	7 days	
OregonR	33.2	13.7	Yes	Yes	Wild type	100	100	13
png[1058]/png[1058]	21.5	10.6	Yes	No	*png* loss	100	92	13
w/w; matα4T GAL4VP16/UASp gnuEGFP	0.1	0.4	No	No	*gnu* gain	71	33	21
png[1058]/png[1058]; matα4T GAL4VP16/UASp gnuEGFP	30.7	12.0	Yes	No	*png* loss	100	100	17
png[1058]/w; matα4T GAL4VP16/UASp gnuEGFP	0.5	0.8	No	No	*gnu* gain	63	8	24
png[1058]/png[1058]; matα4T GAL4VP16/UASp EGFP	24.8	7.8	Yes	No	*png* loss	100	100	12
png[1058]/w; matα4T GAL4VP16/UASp EGFP	28.4	8.0	Yes	Yes	Wild type	100	100	7
w/w; matα4T GAL4VP16/UASp EGFP	30.7	9.4	Yes	Yes	Wild type	100	100	13
matα4T GAL4VP16/w; UASp gnuEGFP/+	None		No	No	*gnu* gain			
matα4T GAL4VP16/w; UASp gnuEGFP plu[6]/plu[6]	Many		Yes	No	*plu* loss			

The *gnu* gain-of-function (gain) phenotype consisted of reduced lifespan, disintegrating ovary (Fig. 5A, Fig. 6D-F), few flaccid eggs were laid and these did not hatch. The *pgn* null (loss) phenotype consisted of normal viability and ovarian morphology (Fig. 5C), many hydrated eggs with giant nuclei (similar to Fig. 4G-I) were laid and these did not hatch. The double mutant combination exhibited the *pgn* loss phenotype. Gnu overexpression combined with a null allele of *plu* resulted in the *plu* loss phenotype.

DISCUSSION

We have mapped the *gnu* gene using chromosome walking and P-element-mediated male recombination and have identified a C to T nonsense mutation in gene CG5272 (Adams et al., 2000) on the *gnu* chromosome. A wild-type copy of this gene rescued the *gnu* mutant phenotype in transgenic *Drosophila*, whereas transformants with the same fragment, but in which the ORF had been mutagenised, did not complement the *gnu* mutation. *gnu* is therefore gene CG5272 and encodes a novel 27 kDa protein with no obvious domains or homologues shared with other organisms.

Gnu phosphorylation

We deduce from its mobility shift on immunoblots that Gnu is phosphorylated before it is needed in oocytes, is dephosphorylated upon egg activation, and that this phosphorylation is independent of Pan gu protein kinase activity. A specific inhibitor of PP1, I-2, stabilised phosphorylated Gnu in vitro, implicating PP1 as the relevant phosphatase. *Drosophila* contains four PP1 genes of which *PP1 87B* provides 80% of total PP1 activity (Dombrádi et al., 1989) and is required for mitotic progression (Axton et al., 1990), though none have previously been ascribed a role in egg activation or fertilisation.

PP1 87B and *PP2A28D* mutations genetically suppress a weak *png* allele (Lee et al., 2001) However, these data are not consistent with our finding that PP1 activates Gnu. If unphosphorylated embryonic Gnu is the active form and Gnu and Pan gu act in the same pathway, then reducing the dose of the activating phosphatase should enhance a *png* phenotype. It may be that these phosphatases oppose *png* action directly by dephosphorylating the Pan gu substrate (Lee et al., 2001) or that they are the cell cycle regulators targeted by Gnu, Plu and PnP as discussed below.

Gain-of-function phenotype

Gnu mis-expression using the UAS-Gal4 system (Rørth, 1998) in *Drosophila* ovaries resulted in sterility due to an inability to lay eggs. Dissection of the ovaries revealed that early oogenesis was unaffected. Gnu was expressed in egg chambers from stage 5 onwards and was localised solely to the nurse cell nuclei. No normal egg chambers could be discerned at stage 10 or later when gross aberrations in the organisation of the actin cytoskeleton resulted in failure to transfer nurse cell cytoplasm into the oocyte. Gnu mobility from such ovaries was identical to the dephosphorylated form suggesting that the protein kinase that phosphorylates Gnu is not present or active in the nurse cells.

Since *gnu*, *plu* and *png* mutations have the same phenotype, we were previously unable to determine whether the gene products act in series or in parallel. Here we have used the dominant ovarian phenotype resulting from Gnu mis-expression to investigate the epistasis of *gnu*, *png* and *plu*. Loss of *png* or *plu* function blocked the ovarian phenotype caused by Gnu mis-expression. This result implies that ectopic Gnu destroys egg chambers only through Pan gu and Plu. It is therefore likely that wild-type Gnu function in the egg and embryo also requires Pan gu. Although Gnu-GFP is more obviously excluded from the larger *png* giant nuclei (Fig. 4H) than from zygotic nuclei (Fig. 4E) we do not favour the explanation that Gnu requires Pan gu for nuclear localisation. Firstly, Gnu-GFP is not specifically nuclear in wild-type embryos (Fig. 4E) and secondly, in a *png* null ovary, ectopic Gnu-GFP is able to concentrate in the polyploid nurse cell nuclei (Fig. 6C). The remaining possibilities are that Gnu acts upstream of Pan gu and Plu or that it acts in a complex with Pan gu and Plu.

We have mis-expressed Gnu in polytene salivary glands and ovarian follicle cells (data not shown). In both cases Gnu was exclusively nuclear, but its expression had no obvious effect on tissue morphology. However, not all nurse cell nuclei in an egg chamber contained Gnu-GFP, suggesting that that the presence of Gnu-GFP reflects the transcriptional activity of the nucleus or depends upon its cell cycle status. Why is Gnu nuclear in polytene cells and cytoplasmic in the diploid syncytial blastoderm and larval neuroblasts (data not shown)? Gnu contains no obvious nuclear import sequence, suggesting that Gnu binds a factor that is cytoplasmic in eggs and embryos (including those with giant nuclei; Fig. 4H), and nuclear in polytene tissues. Polytene and diploid tissues have different Cyclin profiles. Embryos are replete with maternal Cyclins A, B and E whereas polytene tissues have no Cyclin A or B (Lehner and O'Farrell, 1989; Lehner and O'Farrell, 1990; Richardson et al., 1993) and Cyclin E is expressed periodically in nurse cell nuclei and constantly in the

germinal vesicle (Lilly and Spradling, 1996). Our observations fit the Cyclin E pattern, ectopic Gnu was not present in all nurse or follicle cell nuclei and was concentrated in germinal vesicles.

Downstream targets of giant nuclei genes

The DNA replication in *gnu* embryos resembles the endoreduplication observed in *Drosophila* ovarian nurse cells and larval salivary glands and this raises the question of how the normal mechanisms that license DNA replication once per cell cycle are subverted in *gnu* embryos. In yeast, Cdk1 activity, modulated by Cyclin levels, is responsible for resetting replication origins (Hayles et al., 1994) raising the possibility that over-replication in *gnu* embryos may result from inappropriate Cdk1 activity. Indeed, in *gnu*, *plu* or *png* embryos, levels of Cyclin A and B proteins and Cdk1 activity are reduced (Fenger et al., 2000). Restored Cyclin B levels can suppress a weak *png* mutation (Lee et al., 2001).

Several features of early *Drosophila* embryogenesis may have necessitated the evolution of these specialised regulators of the cell cycle. Firstly, distinct cell cycle fates befall the polar body and zygotic nuclei within a common cytoplasm. Secondly, many cell cycle regulators are in excess, so that the first 13 cycles are rapid and lack G_1 or G_2 phases, but S and M phases must alternate accurately. Finally, correct cell cycle regulation is achieved by a small subset of the available cell cycle control proteins (e.g. Cyclins A and B) (Edgar et al., 1994). In this context, Gnu, Plu and Pan gu act coordinately to ensure that the cell cycle oscillations experienced by the nuclei are temporally and locally apt.

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