Maternal Pumilio acts together with Nanos in germline development in *Drosophila* embryos

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The maternal RNA-binding proteins Pumilio (Pum) and Nanos (Nos) act together to specify the abdomen in *Drosophila* embryos. Both proteins later accumulate in pole cells, the germline progenitors. Nos is required for pole cells to differentiate into functional germline. Here we show that Pum is also essential for germline development in embryos. First, a mutation in *pum* causes a defect in pole-cell migration into the gonads. Second, in such pole cells, the expression of a germline-specific marker (*PZ198*) is initiated prematurely. Finally, *pum* mutation causes premature mitosis in the migrating pole cells. We show that Pum inhibits pole-cell division by repressing translation of *cyclin B* messenger RNA. As these phenotypes are indistinguishable from those produced by *nos* mutation, we conclude that Pum acts together with Nos to regulate these germline-specific events.

In many animal groups, maternal factors required for germline formation are localized in a histologically distinguishable region in the egg cytoplasm, or germ plasm, and are inherited in the germline progenitors[1–3]. These maternal factors are believed to direct germline development by regulating a variety of germline-specific cellular events. In *Drosophila*, the germline progenitors known as pole cells are first formed at the posterior pole of the blastoderm embryo. During later embryogenesis, the pole cells pass through the midgut epithelium into the haemocoel and migrate within the embryos to reach the gonads, where they differentiate as functional germ line[4,5]. Expression of zygotic genes is suppressed in pole cells during their migration[6–9]. Cell division is also differentially regulated in pole cells and somatic cells. Pole cells cease mitosis at gastrulation and remain quiescent throughout their migration until they reach the gonads, whereas somatic cells continue to proliferate during the rest of embryogenesis[10]. Although the cellular events during pole-cell migration have been described in detail, it remains unknown how these events, as well as pole-cell migration itself, are controlled by germplasm components.

Among the components of germ plasm, Nos is the only molecule that is known to affect both pole-cell migration and zygotic gene expression in pole cells[8,11,12]. *nos* mRNA, which is enriched in germ plasm, is translated *in situ* to form a posterior gradient of Nos protein in preblastoderm embryos; this gradient acts as a posterior determinant to specify the abdomen[13,14]. Although the Nos gradient becomes undetectable by the cellular blastoderm stage, Nos in germ plasm is partitioned into pole cells and remains detectable throughout pole-cell migration until the pole cells reach the gonads[14]. In the absence of Nos, embryos form pole cells, but they are unable to migrate properly into the embryonic gonads and do not differentiate as functional germ cells[11,12]. In such pole cells, the expression of enhancer-trap markers, which is normally initiated within the gonads, begins prematurely during the migration period[8,11]. Thus, Nos is required to direct germline-specific gene regulation in the migrating pole cells.

Pum protein, which is distributed ubiquitously in the embryo, is a co-factor for Nos function in abdomen formation. Nos, together with Pum, represses translation of maternal *hunchback* (*hb*) mRNA and, consequently, abdomen formation[15–20]. Translational repression of *hb* is mediated by discrete target sites known as *nos*-response elements (NREs) in its 3′ untranslated region (UTR)[21,22]. As Pum binds directly to NREs in a sequence-specific manner, it has been suggested that the interaction of Nos with Pum is essential for translational repression[22,23]. These observations led us to speculate that Pum might also work together with Nos to regulate pole-cell migration and germline-specific events in the migrating pole cells.

Here we report that Pum, like Nos, is essential for pole-cell migration and for gene regulation in the migrating pole cells. In addition, Nos and Pum are both required for the mitotic arrest of the migrating pole cells. These results indicate that Nos and Pum may act together to regulate germline-specific cellular events. We also show that Nos and Pum inhibit mitosis of the migrating pole cells by repressing translation of *cyclin B* mRNA. In contrast, pole-cell migration and gene expression in the pole cells are independent of the translational repression of *cyclin B*.

Results

Function of maternal Pum in early germline development. To determine whether Pum, like Nos, acts in germline development during embryogenesis, we studied the effects of maternal *pum* mutation on the following three germline-specific cellular events. First, we investigated the migratory behaviour of pole cells lacking Pum (*pum* pole cells), because pole-cell migration is autonomously regulated by Nos[11]. We transplanted pole cells formed in embryos lacking Pum (*pum* embryos) into wild-type host embryos. The transplanted *pum* pole cells passed normally through the midgut epithelium into the haemocoel (data not shown). However, none of the transplanted *pum* pole cells were incorporated within the gonads of the hosts (Table 1, Fig. 1b), whereas normal pole cells taken from control embryos were observed in the gonads (Table 1, Fig. 1a). All of the transplanted *pum* pole cells remained in the haemocoel and the gut lumen (Fig. 1b). These results show that Pum is autonomously required in pole cells for their migration into the gonads.

Second, we studied the expression of the enhancer-trap marker

Table 1 The ability of transplanted pole cells to enter into gonads

Genotypes of females producing embryos	No. of transplanted embryos	No. of surviving embryos	Number of embryos with labelled cells	Number of embryos with labelled cells in gonads (%)	Significance
pum^{Msc}/TM3	135	70	42	27 (64)	$P<0.001$
pum^{FC8}/TM3	134	86	35	20 (57)	$P<0.001$
pum^{Msc}/pum^{FC8}	174	82	43	0	

Genetically marked pole cells were transplanted into wild-type host embryos (Oregon-R), and their developmental fates in the hosts were studied. The donor embryos were derived from pum^{Msc}/pum^{FC8}, pum^{Msc}/TM3, or pum^{FC8}/TM3 females mated with PLHΔ23 males. Because all embryonic cells with the PLHΔ23 construct express β-galactosidase after heat treatment[45], we can follow the fate of the transplanted pole cells during embryogenesis by heat treatment and staining for β-galactosidase. After transplantation, the hosts were developed until stage 14–17, and were heat-treated and then stained for β-galactosidase. Significance was calculated by Fisher's exact probability test. Probabilities compared (from top to bottom): pum^{Msc}/TM3 with pum^{Msc}/pum^{FC8}, and pum^{FC8}/TM3 with pum^{Msc}/pum^{FC8}.

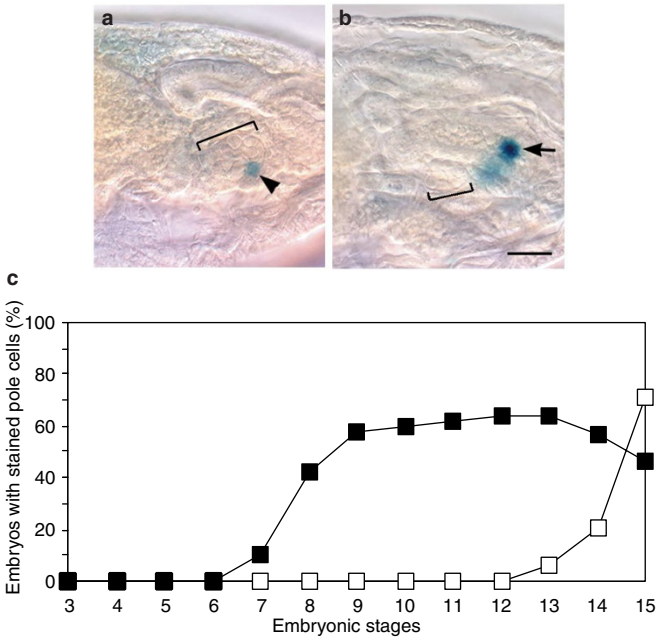

Figure 1 ***pum* activity is essential in pole cells for their migration into the gonads and for gene regulation. a**, **b**, Pole cells were transplanted into wild-type hosts from embryos derived from pum^{Msc}/TM3 (**a**) and pum^{Msc}/pum^{FC8} females (**b**). The transplanted pole cells were identified as blue cells after heat treatment and staining for β-galactosidase (see Methods). The stage-15 embryos carrying the transplanted pole cells were stained with X-gal. The pole cells derived from control embryos (arrowhead in **a**) were incorporated in the gonads (square brackets in **a**, **b**), but none of the pole cells derived from *pum* embryos were incorporated within the gonads. The arrow in **b** indicates a transplanted pole cell in the hindgut lumen. Scale bar represents 20 μm. **c**, *pum* activity is required to prevent the premature expression of *PZ198* during pole-cell migration. Stage-dependent expression of β-galactosidase in pole cells was studied in embryos produced from pum^{Msc}/pum^{FC8} (filled squares) and pum^{Msc}/TM3 (control; open squares) females mated with *PZ198* males. Marker expression in pole cells was examined by double staining with X-gal and an antibody against Vasa protein, a marker for pole cells. The percentage of embryos with stained pole cells is plotted against the embryonic stage. In control embryos, β-galactosidase expression began at stage 13 and reached a maximum in pole cells within the gonads at stage 15 (stages were according to ref. 4). In contrast, β-galactosidase expression was detected prematurely during pole-cell migration (stages 7–12) in *pum* mutant pole cells. The β-galactosidase expression pattern in embryos from pum^{FC8}/TM3 females (data not shown) was indistinguishable from that observed in embryos from pum^{Msc}/TM3 (open squares).

PZ198 in *pum* pole cells. *PZ198* expression, which is normally initiated in pole cells within the gonads, begins prematurely during pole-cell migration in embryos lacking Nos (*nos* embryos)[8,11]. Similarly, *PZ198* expression began prematurely, at stage 7, in *pum* mutant pole cells, as compared with stage 13 in control embryos (Fig. 1c). Thus Pum is also required to repress the premature expression of the enhancer-trap marker in pole cells.

Finally, we analysed the effect of *pum* and *nos* mutations on cell-cycle arrest during pole-cell migration. In normal development, pole cells remain quiescent in G2 phase of the cell cycle during stages 7–15 (refs 10, 24–26). We expected that Nos and Pum repress the entry of pole cells into mitosis, because pole cells initiate cell division just after Nos becomes undetectable in pole cells at stage 15. To monitor the cell cycle in pole cells, we used antibodies against a phosphorylated form of histone H3 (PH3) and cyclin E. PH3 is detectable in mitosis but is absent during interphase[27], whereas cyclin E is expressed specifically in S and G2 phases[28]. As described below, we found that the disappearance of cyclin E from pole cells was linked to cell-cycle progression from G2 to G1 phase, whereas cyclin E is not degraded during cell cycling of somatic cells[29]. Consistent with the previous observation that migrating pole cells in wild-type embryos are arrested in G2 phase[26,28], almost all pole cells in stage 7–15 embryos showed cyclin E staining (Fig. 2e), but not PH3 staining (Fig. 2a,d). In contrast, in *pum* and *nos* embryos, the percentage of pole cells expressing cyclin E gradually decreased during stages 7–15 (Fig. 2e), and PH3-positive pole cells became detectable during these stages (Fig. 2b–d). Thus, the mutant pole cells are prematurely released from G2 arrest and enter into mitosis. Taken together, these observations show that Pum and Nos are both required for the repression of the G2/M transition in the migrating pole cells.

These results, together with those of ref. 11, show that the *pum* mutant phenotype is indistinguishable from that of *nos* mutants in early germline development. Thus we conclude that Pum acts with Nos to regulate germline-specific cellular events.

Pum and Nos inhibit pole-cell division by repressing cyclin B production. The next question we asked is how Pum and Nos regulate these germline-specific cellular events. Considering that both proteins suppress the translation of NRE-containing mRNAs in somatic cells[20,22,23], we expected that Pum and Nos repress the translation of an mRNA(s) that encodes a regulatory factor(s) responsible for the germline-specific cellular events. One candidate target molecule is maternal *cyclin B* mRNA. This mRNA is localized in germ plasm and partitioned into pole cells, but its translation is repressed until stage 15 (ref. 30). Furthermore, maternal *cyclin B* mRNA contains an NRE-like element within its 3′ UTR[30].

To investigate whether both Pum and Nos are required to repress the production of cyclin B protein in migrating pole cells, we stained *pum* and *nos* embryos with an antibody against cyclin B. In control embryos, cyclin B started to accumulate in pole cells at stage 14 (Fig. 3a–c, j). In contrast, in the mutant embryos, cyclin B was prematurely expressed in pole cells during stages 5–13 (Fig. 3d–j). In these embryos, almost all pole cells expressed cyclin B (Fig. 3). These results show that Pum and Nos repress the production of cyclin B in pole cells during their migration to the gonads.

The above observation led to the idea that the repression of cyclin B production in the migrating pole cells causes their cell-cycle arrest in G2 phase, because cyclin B is a factor required for the G2/M transition[31,32]. To investigate this possibility, we determined

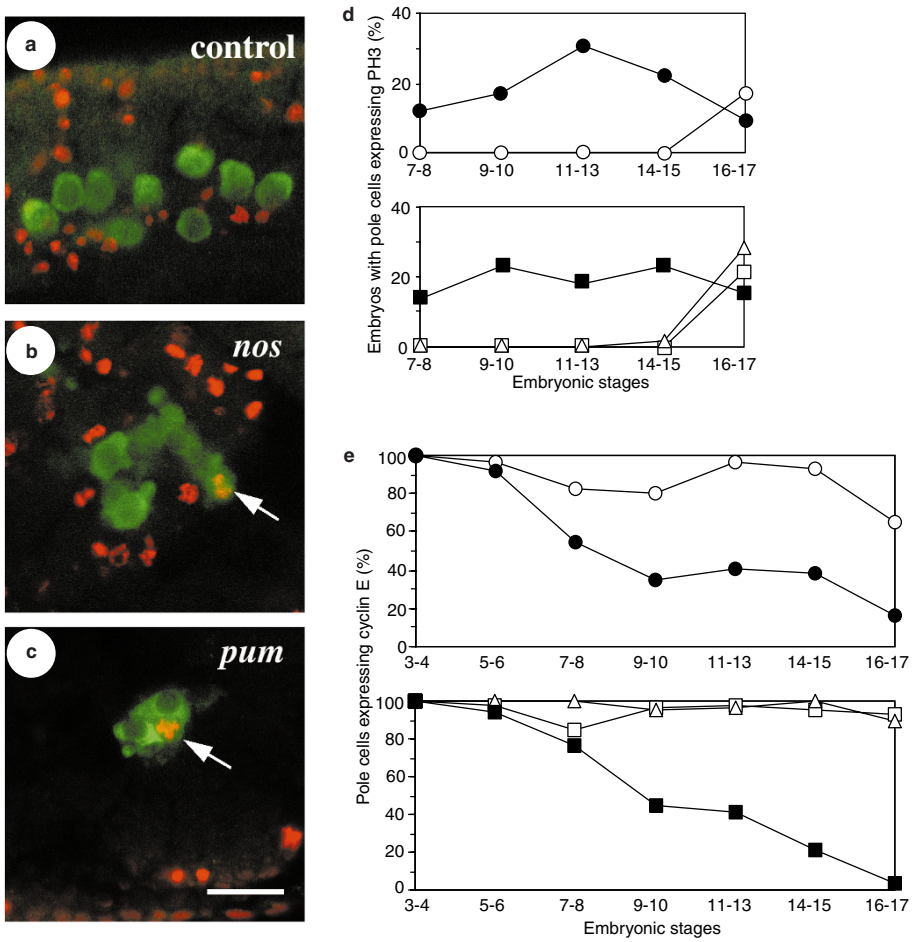

Figure 2 ***nos* and *pum* activities are both required to prevent pole-cell division during pole-cell migration. a–c**, Confocal images of migrating pole cells in embryos derived from nos^{BN}/TM3 (**a**), nos^{BN}/nos^{BN} (**b**) and pum^{Msc}/pum^{FC8} females (**c**). The embryos at stage 12 were double-stained with antibodies against PH3 (red) and Vasa (green). Arrows point to PH3-positive pole cells. Scale bar represents 20 μm. **d**, Stage-dependent expression of PH3 in pole cells of embryos derived from nos^{BN}/TM3 (open circles), nos^{BN}/nos^{BN} (filled circles), pum^{Msc}/TM3 (open squares), pum^{FC8}/TM3 (open triangles) and pum^{Msc}/pum^{FC8} (filled squares) females. The percentage of embryos with PH3-positive pole cells is plotted against embryonic stage. The number of embryos examined at each stage was 11–80 (43 on average). One to three pole cells per embryo showed PH3 staining. **e**, Stage-dependent expression of cyclin E in pole cells. Embryos were stained with an anti-cyclin-E antibody. The percentage of cyclin-E-positive pole cells is plotted against embryonic stage. Symbols in **e** are as in **d**. The number of pole cells counted at each stage was 15–578 (158 on average).

Table 2 Expression of *PZ198* in pole cells of *cyclin B–nos 3′ UTR* embryos

Genotypes of females producing embryos*	Percentage of embryos with pole cells expressing β-galactosidase† (Total number of embryos scored) Stages 7–13	Stages 14–17
yw	0 (74)	55.9 (118)
cyclin B–nos 3′ UTR6	6.1 (33)‡	54.8 (93)
cyclin B–nos 3′ UTR5	0 (47)	84.0 (25)

* *yw* females with or without the *cyclin B–nos 3′* UTR construct were mated with males from the enhancer-trap line *PZ198*, and the marker expression in pole cells was examined in their progenies.
† Embryos at stages 7–13 and at stages 14–17 were double-stained for β-galactosidase and Vasa. The percentages of embryos with pole cells expressing β-galactosidase are shown.
‡ Only two of these embryos showed *PZ198* expression in pole cells at stage 12/13. Even in normal embryos, *PZ198* expression was only occasionally seen at stage 12/13 (Fig. 1c).

Table 3 Number of pole cells in mutant embryos

Genotypes of females producing embryos*	Average number of pole cells in an embryo (no. of embryos studied)†	Significance‡
nos^{BN}/TM3	29.5 (11)	$P<0.001$
nos^{BN}/nos^{BN}	6.3 (45)	
pum^{Msc}/TM3	30.8 (11)	$P<0.001$
pum^{FC8}/TM3	24.7 (9)	$P<0.001$
pum^{Msc}/pum^{FC8}	5.2 (9)	
yw	18.8 (29)	
cyclin B–nos 3′ UTR6	25.6 (25)	$P<0.001$
cyclin B–nos 3′ UTR5	31.6 (9)	$P<0.001$

* *nos/nos*, *nos*/TM3, *pum/pum*, *pum*/TM3 females, and *yw* females with or without the *cyclin B–nos 3′* UTR construct were mated with Oregon-R males, and the numbers of total pole cells were counted in their progenies.
† Embryos at stages 14–17 were stained with an antibody against Vasa protein. Pole cells were identified as the cells with Vasa staining. Total numbers of Vasa-positive pole cells within and outside gonads were counted. The average numbers of Vasa-positive cells in an embryo are shown.
‡ Probability was calculated by Fisher's exact probability test. Probabilities compared (from top to bottom): nos^{BN}/TM3 with nos^{BN}/nos^{BN}; pum^{Msc}/TM3 with pum^{Msc}/pum^{FC8}; pum^{FC8}/TM3 with pum^{Msc}/pum^{FC8}; *yw* with *cyclin B–nos 3′* UTR6; *yw* with *cyclin B–nos 3′* UTR5.

whether the misexpression of cyclin B in quiescent pole cells is sufficient to drive these cells into mitosis. We expressed RNA encoding cyclin B under the control of the *nos* promoter, and localized it to polar plasm and pole cells using the *nos* 3′ UTR[9] (E. R. Gavis, personal communication). As the 3′ UTR of *cyclin B* mRNA is replaced

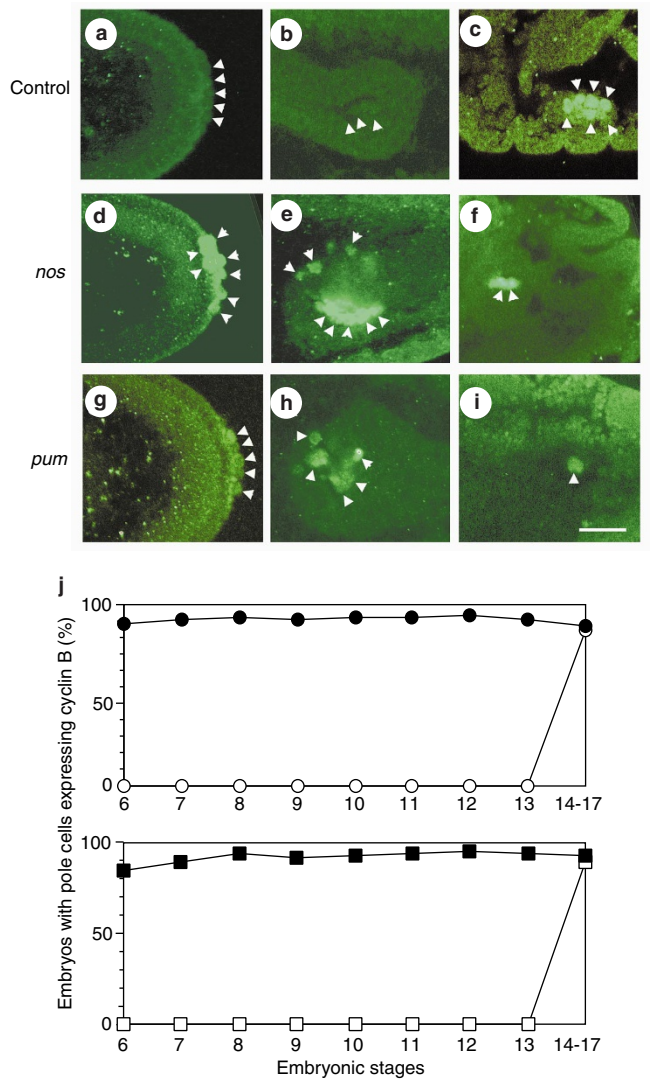

Figure 3 ***nos* and *pum* activities are both required to prevent premature expression of cyclin B in pole cells. a–i**, Confocal images of pole cells in embryos derived from nos^{BN}/TM3 (**a–c**), nos^{BN}/nos^{BN} (**d–f**) and pum^{Msc}/pum^{FC8} (**g–i**) females. **a**, **d**, **g**, Blastodermal embryos at stage 5; **b**, **e**, **h**, embryos at stage 9; **c**, **f**, **i**, embryos at stage 15. Embryos were stained with antibodies against cyclin B (**a–i**) and Vasa (data not shown). Arrowheads show Vasa-positive cells. Scale bar represents 20 μm. Premature expression of cyclin B was observed at stage 5 in the mutant pole cells. **j**, Stage-dependent expression of cyclin B in pole cells. The percentage of embryos with cyclin-B-positive pole cells is plotted against the stage. Symbols in **j** are as shown in Fig. 2d. 3–46 embryos (average 14) were studied at each stage.

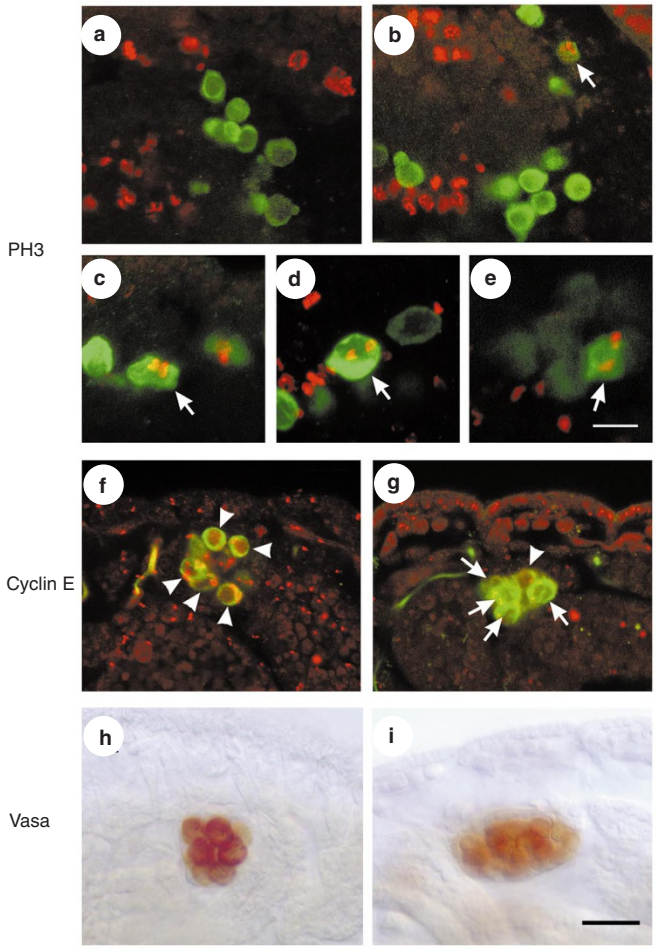

Figure 4 **Induction of cyclin B expression is able to drive quiescent pole cells into mitosis. a–g**, Expression of PH3 (**a–e**) and cyclin E (**f**, **g**) in pole cells of embryos derived from females with (**b–e**, **g**) and without (**a**, **f**) the *cyclin B–nos* 3′ UTR. **a–e**, Embryos at stages 10–11 were double-stained with antibodies against PH3 (red) and Vasa (green). Arrows in **b–e** show PH3-positive pole cells at prophase (**b**), metaphase (**c**), and anaphase/telophase (**d**, **e**). 10–20% of the PH3-positive pole cells were in metaphase and anaphase/telophase. **f**, **g**, Embryos at stage 16 were double-stained with antibodies against cyclin E (red) and Vasa (green). Arrowheads and arrows show pole cells with and without cyclin E staining, respectively. **h**, **i**, Gonads in embryos derived from females with (**i**) and without (**h**) the *cyclin B–nos* 3′ UTR construct. Embryos at stage 17 were stained with an anti-Vasa antibody. In both embryos, pole cells were normally incorporated within the gonads. An increased number of pole cells was incorporated within gonads in embryos derived from females with the *cyclin B–nos* 3′ UTR construct (**i**), compared with that in the embryos from females without this construct (**h**). Embryos derived from females with *cyclin B–nos* 3′ UTR developed normally, in that they became viable and fertile adults, despite aberrant cell-cycle regulation of pole cells (see text). **a**, **b**, **f–i** are at the same magnification. Scale bars in **e** (for **c–e**) and **i** (for **a**, **b**, **f–i**) represent 20 μm.

by the *nos* 3′ UTR, the resulting mRNA produces cyclin B in the migrating pole cells (data not shown).

In embryos produced from females with two copies of the *cyclin B–nos* 3′ UTR fusion gene, pole cells prematurely entered mitosis, as assessed by PH3 staining (Fig. 4). PH3-positive pole cells were observed in 10.3% of the embryos (N (number of embryos studied) = 78 at stages 8–14), a significant increase from 0% ($N = 176$, $P<0.001$, Fisher's exact probability test) obtained with control embryos from females without the fusion gene (Fig. 4a,b). PH3-positive pole cells were clearly identifiable as mitotic cells at prophase (Fig. 4b), metaphase (Fig. 4c) and anaphase/telophase (Fig. 4d,e), confirming that migrating pole cells in *cyclin B–nos* 3′ UTR embryos progress through mitosis.

The percentage of cyclin-E-positive pole cells at stages 16–17 was significantly decreased in *cyclin B–nos* 3′ UTR embryos (40.0%; n (number of pole cells studied) = 30) compared with control embryos (75.3%; $n = 291$; $P<0.001$, Fisher's exact probability test) (Fig. 4f, g). To determine whether pole cells without a cyclin E signal are in a postmitotic state in G1 phase, we counted the DNA content in cyclin-E-negative and -positive pole cells by DNA fluorescent cytometry (see Methods). In control embryos, the values of the DNA fluorescence in the cyclin-E-negative and -positive pole cells relative to those in somatic cells at G1 phase were 1.12 and 2.05, respectively ($P<0.01$, U-test; cyclin-E-negative, $n = 5$; cyclin-E-

positive, $n=16$). In *cyclin B–nos* 3′ UTR embryos, the relative values of cyclin-E-negative and -positive pole cells were 1.01 and 1.97, respectively ($P<0.01$, *U*-test; cyclin-E-negative, $n=12$; cyclin-E-positive, $n=10$). These results indicate that the disappearance of cyclin E from pole cells is intimately linked to cell-cycle progression from G2 to a postmitotic state in G1 phase. These results show that the induction of cyclin B is able to drive pole cells through mitosis into G1 phase. We conclude that Pum and Nos inhibit the transition from G2 phase to mitosis in the migrating pole cells by repressing cyclin B production.

To determine whether the repression of cyclin B by Pum and Nos is required for other germline-specific cellular events, we studied the effect of cyclin B misexpression in pole cells on their migration and on *PZ198* expression. In *cyclin B–nos* 3′ UTR embryos, pole cells colonized the gonads normally (Fig. 4h, i). The average number of pole cells within a gonad was 12.1 in *cyclin B–nos* 3′ UTR embryos ($N=50$), 9.3 in control embryos ($N=58$) and 7.5 in embryos from females carrying a *nos* promoter–3′ UTR construct that lacks *cyclin B* ($N=35$). *PZ198* expression was normal in pole cells of *cyclin B–nos* 3′ UTR embryos (Table 2). These observations indicate that cyclin B may not be the only regulatory target for Pum and Nos in pole cells.

During these experiments, we noted another role of Pum and Nos in early germline development. In *cyclin B–nos* 3′ UTR embryos, the number of pole cells was increased compared with that observed in the controls (Table 3). This increase in pole-cell number could result from the premature mitosis of the pole cells. However, in *pum* and *nos* embryos, the number of Vasa-positive pole cells was significantly less than that in the control embryos (Table 3), although the mutant pole cells entered into mitosis, albeit prematurely (Fig. 2). These results suggest that a significant fraction of pole cells were lost in the mutant embryos. Thus, Pum and Nos may ensure the survival of pole cells by a pathway independent of cyclin B repression.

Discussion

Pum and Nos are required for a variety of germline and somatic developmental processes[11,12,33–35]. Whereas Pum and Nos cooperate with each other in the pathway leading to abdominal formation[20], they appear to act separately during early oogenesis[12,35], as *pum* affects germline stem-cell maintenance, whereas *nos* affects a later step, germline cyst development[12,35]. High levels of Pum and Nos are detected in germline stem cells and cystoblasts, respectively[12]. These differences indicate that Nos and Pum may interact with different partners during oogenesis. In contrast to their mode of action during oogenesis, however, *pum* and *nos* mutations lead to essentially identical phenotypic spectra in embryonic germline development. As *pum* mutations have no effect on the expression, distribution or stability of Nos, and vice versa[20], we conclude that Pum acts in conjunction with Nos in early germline development.

pum and *nos* mutations cause the premature expression of cyclin B protein in pole cells. As these mutations do not affect posterior localization of maternal *cyclin B* mRNA or its partitioning into pole cells (data not shown), we conclude that translation of *cyclin B* mRNA is usually repressed by Pum and Nos in pole cells. *Cyclin B* mRNA contains an NRE-like sequence in its 3′ UTR, called the translation-control element (TCE)[30]. Deletion of the TCE from the 3′ UTR of an epitope-tagged *cyclin B* mRNA results in a phenotype similar to that caused by *nos* and *pum* mutations[30] (Fig. 3). These observations lead us to conclude that Pum/Nos-dependent translational repression of *cyclin B* mRNA is mediated by the TCE. Given that Pum binds to the NRE *in vitro*[23], it is reasonable to suggest that Pum binds directly to the TCE. This is, to our knowledge, the first demonstration that maternal factors regulate the translation of specific mRNA in germline progenitors.

Our results indicate that Pum and Nos may inhibit mitosis of the migrating pole cells by repressing translation of maternal *cyclin B* mRNA. During normal development, pole-cell division begins after Nos is degraded in pole cells within the gonads at stage 15 (refs 10, 14). However, this does not necessarily mean that translation of maternal *cyclin B* triggers the onset of mitosis, because cyclin B[32] and *cyclin B3* (ref. 32) are zygotically expressed in pole cells within the gonads. It is likely that zygotic expression of these factors may be essential to initiate pole-cell division within the gonads.

Coexpression of cyclin B with a constitutively active Cdc2 (Cdc2AF) drives most pole cells into mitosis simultaneously[26]. However, cyclin B or Cdc2AF alone induces mitosis less efficiently than do both proteins together[26] (Fig. 4). Thus, misexpression of cyclin B is able to drive quiescent pole cells into mitosis, but is insufficient to induce efficient mitosis. Presumably, quiescence of pole cells in G2 phase results from limitations in the amounts of cyclin B and active Cdc2, and increased levels of cyclin B may recruit a small amount of active Cdc2 to initiate pole-cell division.

The prematurely induced mitosis in pole cells lacking Pum or Nos is not followed by S phase, as shown by the failure of the mutant pole cells to reaccumulate cyclin E after mitosis (Fig. 2e). Similar results are obtained by misexpression of cyclin B in pole cells. These results indicate that pole cells may be arrested in G1 phase after mitosis, and that this arrest is independent of Pum/Nos function. Thus, continued and sequential cell cycling of the migrating pole cells is blocked through multiple cell-cycle checkpoints.

This argument leads us to speculate that the inhibition of sequential cell cycling has an important role in early germline development. One possible role is to prevent the dilution of maternal factors incorporated in pole cells. Pum and Nos may repress the G2/M transition to keep their concentration high enough for pole-cell migration and zygotic gene regulation. The quiescence of pole cells may be ensured by their arrest in G1 phase by other maternal factor(s). It is interesting that germline progenitors cease mitosis in nematode embryos, in which germline development is regulated by maternal factors[36,37]. In contrast, proliferation of germline progenitors continues in mouse embryos, in which the maternal contribution to germline development is less evident[38].

Our results show that pole-cell migration is unaffected by either the misexpression of cyclin B or the single round of mitosis that results. This suggests that pole-cell migration is independent of cell-cycle regulation by Pum and Nos. Presumably, Pum and Nos could repress the translation of a maternal transcript(s) other than *cyclin B* in pole cells, a transcript that otherwise prevents pole-cell migration.

nos and *pum* pole cells fail to migrate properly after their passage through the midgut epithelium into haemocoel[11,12]. Gene regulation is also altered in the mutant pole cells after their exit from the midgut pouch; they prematurely express enhancer-trap markers that are normally activated within the gonads (Fig. 1). Thus the failure of the mutant pole cells to migrate properly could result from the premature gene expression in these cells. The premature gene expression may mean that these pole cells behave as if they have already completed their migration to the gonads, and can be explained by the failure of Nos and Pum to repress the production of a regulatory factor(s) responsible for the enhancer activation[8]. Both proteins may directly repress the translation of mRNAs encoding the activator for the enhancers.

The conservation of RNA-binding domains in Pum and Nos in vertebrates and invertebrates indicates that they may have a widespread and central role in germline development[39–41]. On the other hand, the Pum/Nos function in abdominal formation may be subsidiary, because embryos lacking both maternal *nos* and *hb* mRNAs form abdomen[16–18]. These arguments are further supported by the observations that mRNA encoding a Nos-related protein is associated with the germ line in a variety of animals. In the diverse dipteran insects and *Xenopus* embryos, *nos*-related mRNAs are localized in the germ plasm and are later found in the germline progenitors[40,42,43]. More important, the ability of Pum to bind NREs, as well as the NRE sequence itself, appears to be conserved. The RNA-binding domain of human Pum is able to bind to the *hb* NREs

in a sequence-specific manner[41]. In nematodes, an NRE-like sequence is observed in *glp-1* mRNA, and its translation is repressed in germline progenitors[44]. On the basis of these observations, we propose that translational regulation by Pum and Nos may be essential for germline development in many animal groups. Our data provide an important first step towards understanding the regulatory mechanism of germline development. □

Methods

Fly stocks.

A combination of strong *pum* alleles, *In(3R)Msc* and *T(1;3)FC8*, was used as a source of *pum* embryos. Females carrying *In(3R)Msc* (pum^{Msc}) and *T(1;3)FC8* (pum^{FC8}) in *trans* produce progenies that lack detectable Pum protein and show a strong maternal *pum* phenotype[20,22]. The *nos* allele used is nos^{BN}. Females homozygous for nos^{BN} express *nos* RNA in the germarium but not later during oogenesis, such that maternal *nos* RNA is not deposited into the egg[12]. *PZ198* was as described[11].

Pole-cell transplantation.

Pole-cell transplantation was done as described[11]. Pole-cell-donor embryos were derived from pum^{Msc}/pum^{FC8}, pum^{Msc}/TM3 or pum^{FC8}/TM3 females mated with PLHΔ23 males[45]. Because all embryonic cells with the PLHΔ23 construct express β-galactosidase after heat treatment, we can follow the fate of the transplanted pole cells during embryogenesis by heat treatment and staining for β-galactosidase[45]. Host embryos were derived from wild-type (Oregon-R) females.

Double-staining with X-gal and antibody.

Embryos were fixed and stained with X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (Wako, Osaka, Japan) as described[45]. The stained embryos were transferred onto double-sided sticky tape, covered with 100% methanol, and devitellinized with a tungsten needle. They were then blocked with 2.5% goat serum (Vector Laboratory) in BA+T buffer (0.2% Tween 20 in Block Ace (Dainippon Pharmaceutical, Osaka, Japan) for 1h, and incubated with a rabbit anti-Vasa antibody (1:200 dilution; a gift from P. Lasko) followed by a biotinylated secondary antibody (1:200 dilution, Vector Laboratory) as described below. Signal detection was carried out with vectastain ABC Elite kit (Vector Laboratory) and Histo Mark Orange (Kirkegaard & Perry Laboratories, Gaithersburg, MD).

Immunostaining.

Immunofluorescent staining of embryos was done as described[8]. To increase the permeability of antibodies into embryos, the fixed and devitellinized embryos were nicked in their anterior portions with a tungsten needle.

When the embryos were double-stained for PH3 and Vasa, or for cyclin E and Vasa, primary antibodies were also incubated simultaneously with the embryos in BA+T buffer for 1–3 days at 4 °C. A rabbit anti-PH3 antibody (1:200 dilution, Upstate Biotechnology), a rat anti-cyclin-E antibody (1: 100 dilution; a gift from H. Richardson) and a chick anti-Vasa antibody (1: 10,000 dilution; a gift from K. Howard) were used. Two secondary antibodies were incubated simultaneously with the embryos in 10% BA+T buffer (10% Block Ace, 0.2% Tween 20) containing 2.5% goat serum overnight at 4 °C. Secondary antibodies were a Texas-red-conjugated anti-rabbit IgG (1:50 dilution, Jackson Immuno Research), a Texas-red-conjugated anti-rat IgG (1:50 dilution, Cappel), and a fluorescein isothiocyanate (FITC)-conjugated anti-chick IgG (1:10 dilution, Kirkegaard & Perry Laboratories). The stained embryos were washed in washing buffer (0.2% Tween 20 in PBS) twice for 10 min each time, mounted in Vectashield (Vector Laboratory), and then observed under a confocal laser microscope (Leica).

When embryos were double-stained for cyclin B and Vasa, the embryos were first incubated with a mouse anti-cyclin-B antibody (1:4 dilution; a gift from C. Lehner) alone overnight at 4 °C, then incubated with an FITC-conjugated secondary antibody (1:20 dilution, Cappel) overnight at 4 °C. Subsequently, the embryos were incubated with a rabbit anti-Vasa antibody (1:200 dilution) overnight at 4 °C, followed by incubation with a Texas-red-conjugated secondary antibody (1:20 dilution, Amersham) overnight at 4 °C.

To study the number of pole cells, the embryos were incubated with a rabbit anti-Vasa antibody (1:200 dilution) overnight at 4°C. Biotinylated secondary antibody (1:200 dilution) was used. Signal detection was carried out using the ABC Elite kit as above.

Construction of *cyclin B–nos* 3′ UTR gene and germline transformation.

A *nos* promoter cassette encompassing 750 base pairs (bp) of *nos* promoter and *nos* 5′ UTR was fused to a *nos* 3′ UTR cassette containing the entire *nos* 3′ UTR and ~75 bp of 3′ flanking region by introducing an *Eco*RI fragment from pBS-KS nos3′UT (a gift from E. Gavis) into pBS-KS Pnos (a gift from E. Gavis) at the *Eco*RI site. In this pBS-Pnos-nos3′UT vector, a unique *Nde*I site was introduced to create an AUG at the position of the intact initiation codon of the *nos* gene (E. Gavis, personal communication). To construct the *cyclin B–nos* 3′ UTR transgene, the *cyclin B* coding region was amplified by performing a polymerase chain reaction with the following primers: 5′-CATATGGTGGGCACAACACTGAAAATG (*cyclin B* 5′ primer) and 5′-CATATGCTATTTCCTCTGGCTCTGGCC (*cyclin B* 3′ primer) (underlined sequences are *Nde*I sites), using the *cyclin B* complementary DNA as a template. After the integrity of amplified *cyclin B* cDNA was confirmed by DNA sequencing, this *Nde*I–*Nde*I fragment was subcloned into the pBS-Pnos-nos3′UT vector. Subsequently, the *Bam*HI–*Kpn*I fragment, which contains the entire Pnos-*cyclin B–nos* 3′ UTR hybrid gene, was subcloned into pCaSpeR4 (ref. 46) to make the plasmid for transformation.

Germline transformation was done as described in ref. 47 using *yw* embryos as recipients. Two independent w^+ transformants were in-bred to establish homozygous stocks, *cyclin B–nos* 3′ UTR5 and *cyclin B–nos* 3′ UTR6. We describe data obtained with one of the two transformant lines (*cyclin B–nos* 3′ UTR6) in the text, as we found no significant difference between the two lines.

DNA quantification.

Embryos at stage 15–16 were double-stained with a chick anti-Vasa antibody and a rat anti-cyclin-E antibody as described above. Secondary antibodies used were an FITC-conjugated anti-chick IgG and a Cy5-conjugated anti-rat IgG (1:60 dilution, Jackson Immuno Research). The stained embryos were incubated in washing buffer containing 100 μg ml^{-1} RNase A for 15 min at 37 °C. They were then stained in 10% BA+T buffer containing 10 μg ml^{-1} propidium iodide (Sigma) for 30 min, and mounted in Vectashield. Under a confocal laser microscope (Leica), serial optical sections (0.7 μm) through gonads were obtained (about seven sections per pole-cell nucleus). To quantify DNA fluorescence, fluorescence intensities from the area occupied by the nucleus were first determined using TCS-NT software (Leica) for each section, and then summed up. For each embryo, the nuclei from 1–3 pole cells with or without cyclin E signal and from three somatic cells at G1 phase were randomly selected in the same focal plane. We used fat-body cells as somatic G1 cells, because they complete their mitosis at stage 11 and become polyploid after embryogenesis[48]. The fluorescence intensities thus measured were consistent for each class of nuclei in an embryo but were variable from embryo to embryo. For this reason, the values of DNA fluorescence were first normalized to that of the somatic G1 nuclei (set at 1.00) for each embryo, then the average value of 4–6 embryos was calculated. Significance between the values obtained from the pole cells with and without cyclin E signal was calculated by Mann–Whitney *U*-test.

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