The Drosophila \textit{pumilio} Gene Encodes Two Functional Protein Isoforms That Play Multiple Roles in Germline Development, Gonadogenesis, Oogenesis and Embryogenesis

Michael Parisi and Haifan Lin

Department of Cell Biology, Duke University Medical Center, Durham, North Carolina 27710

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ABSTRACT

The \textit{pumilio} (\textit{pum}) gene plays an essential role in embryonic patterning and germline stem cell (GSC) maintenance during oogenesis in Drosophila. Here we report on a phenotypic analysis using \textit{pum} \textit{mutante} mutations, which reveals multiple functions of \textit{pum} in primordial germ cell proliferation, larval ovary formation, GSC division, and subsequent oogenic processes, as well as in oviposition. Specifically, by inducing \textit{pum} \textit{mutante} GSC clones at the onset of oogenesis, we show that \textit{pum} is directly involved in GSC division, a function that is distinct from its requirement in primordial germ cells. Furthermore, we show that \textit{pum} encodes 156- and 130-kD proteins, both of which are functional isoforms. Among \textit{pum} \textit{mutante} mutations, \textit{pum} \textit{mutante} specifically eliminates the 156-kD isoform but not the 130-kD isoform, while \textit{pum} \textit{mutante} and \textit{pum} \textit{mutante} specifically affect the 130-kD isoform but not the 156-kD isoform. Normal doses of both isoforms are required for the zygotic function of \textit{pum}, yet either isoform alone at a normal dose is sufficient for the maternal effect function of \textit{pum}. A \textit{pum} cDNA transgene that contains the known open reading frame encodes only the 156-kD isoform and rescues the phenotype of both \textit{pum} \textit{mutante} and \textit{pum} \textit{mutante} mutants. These observations suggest that the 156- and 130-kD isoforms can compensate for each other's function in a dosage-dependent manner. Finally, we present molecular evidence suggesting that the two PUM isoforms share some of their primary structures.

During subsequent larval development, both germline and somatic cells in the female gonad proliferate without detectable differentiation, so that by the third instar larval stage, there are \textasciitilde55 pregermline stem cells in each larval ovary that occupy the medial portion of the ovary and are flanked by apical and basal somatic cells (King 1970; see also Table 1 and Figure 1A).

The late third instar larval stage marks the completion of preoogenic development and the onset of ovary differentiation and oogenesis. At this stage, one group of the anterior somatic cells differentiates into terminal filaments. A second group migrates between terminal filaments, partitioning the ovary into \textasciitilde17 identical units called germaria, while a third group migrates to the posterior to form the basal stalk cells of the germarium (King 1970; Godt and Laski 1995). Concomitant to ovarian differentiation, oogenesis is initiated, producing differentiated daughter cells called cystoblasts. Among the three to four pregermline stem cells in each ovariole, two to three become mature stem cells and undergo self-renewing, asymmetric divisions (King 1970; Schupbach et al. 1978; Wieschaus and Szabad 1979; Bhat and Schedl 1997; Deng and Lin 1997; Lin and Spradling 1997). As oogenesis proceeds, each germarium produces a continuous string of developing egg chambers, each of which represents the product of a single germline stem cell division. Each egg chamber contains a posteriorly located developing oocyte inter-
connected by cytoplasmic bridges to 15 polytenized nurse cells. This cyst of germline cells is enveloped by a monolayer of follicle cells involved in providing maternal supply as well as patterning of the future egg. By the end of oogenesis, nurse cells and follicle cells degenerate. The mature oocyte is now ready for fertilization.

Germline development and oogenesis have been analyzed extensively at the molecular level. Genes required for the initial establishment of germline cells, their subsequent migration, gonadogenesis, and various stages of oogenesis have been identified (reviewed in Manseau and Schupbach 1989; Schupbach and Roth 1994; Wei and Mahowald 1994; Williamson and Lehmann 1996; Lin 1998). Molecular analyses have revealed the importance of transcriptional regulation, mRNA localization, translational regulation, cell-cell signaling, and cytoskeletal mechanisms in germline specification and development.

Among the known molecular mechanisms, pumilio appears to play a crucial role in germline stem cell maintenance during oogenesis (Lin and Spradling 1997; Forbes and Lehmann 1998). pumilio (pum) was originally identified as a maternal effect gene required for the posterior patterning of the Drosophila embryo (Nüsslein-Volhard et al. 1987). Molecular analyses of pum showed that it encodes a 156-kD protein from a transcription unit >160 kb in size (Barker et al. 1992; MacDonald 1992; measured as 165 kD by Murata and Wharton 1995). During early embryogenesis, pum interacts with another posterior group gene, nanos (nos), to suppress the translation of the maternally loaded hunchback (hb) transcription factor in the posterior region of the embryo (Hulskamp et al. 1989; Irish et al. 1989; Struhl 1989). The PUM protein achieves this suppression by binding to the nos response elements in the 3'-untranslated region of hb mRNA (Murata and Wharton 1995). This binding appears to promote deadenylation of the hb mRNA, causing its destabilization (Wreden et al. 1997). Analysis of the PUM protein has shown that a 334-amino-acid region near the C terminus binds maternal hb mRNA (Zamore et al. 1997, 1999; Wharton et al. 1998). These experiments established PUM as a new RNA-binding molecule that functions as a translational repressor. PUM also appears to regulate the proper asymmetric division and maintenance of germline stem cells during oogenesis, even though it is not known whether PUM functions as a translational repressor in this process (Lin and Spradling 1997; Forbes and Lehmann 1998). The PUM protein is present in germline stem cells at a higher concentration than in the cystoblast; various pum mutations affect either the mitotic ability or the divisional asymmetry of germline stem cells, leading to the depletion of the germline during oogenesis. These observations point to a zygotic role of pum in germline stem cell division.

Here we report a systematic study of the zygotic function of pum during germline development, gonadogenesis, and oogenesis. Our phenotypic and clonal analyses reveal multiple functions of pum as a zygotic gene that acts cell autonomously in preoogenic development, ovarian morphogenesis, germline stem cell division, and subsequent oogenesis. Moreover, we show that pum encodes at least two major functional protein isoforms, the known 156-kD isoform and a novel 130-kD isoform. Either isoform alone is sufficient for the maternal effect function of pum, yet neither one alone is sufficient for its zygotic function. This insufficiency, however, can be compensated for in a dosage-dependent manner. These observations reveal novel functions of pum during development and the complexity in the modulation of pum activity.

MATERIALS AND METHODS

Drosophila strains and culture: All Drosophila strains were grown at 24°C on yeast-containing corn meal/molasses medium. Canton-S and Oregon-R strains were used as wild-type flies. P-element insertion mutations in pum that cause germline stem cell defects, known as the ovarite (ovt) class of pum alleles, such as pum2003, pum689, pum207, pum206, pum508, and pum509, were originally described by Lehmann and Nüsslein-Volhard (1987) and listed in Flybase (Irish et al. 1995). During early embryogenesis, pum interacts with another posterior group gene, nanos (nos), to suppress the translation of the maternally loaded hunchback (hb) transcription factor in the posterior region of the embryo (Hulskamp et al. 1989; Irish et al. 1989; Struhl 1989). The PUM protein achieves this suppression by binding to the nos response elements in the 3'-untranslated region of hb mRNA (Murata and Wharton 1995). This binding appears to promote deadenylation of the hb mRNA, causing its destabilization (Wreden et al. 1997). Analysis of the PUM protein has shown that a 334-amino-acid region near the C terminus binds maternal hb mRNA (Zamore et al. 1997, 1999; Wharton et al. 1998). These experiments established PUM as a new RNA-binding molecule that functions as a translational repressor. PUM also appears to regulate the proper asymmetric division and maintenance of germline stem cells during oogenesis, even though it is not known whether PUM functions as a translational repressor in this process (Lin and Spradling 1997; Forbes and Lehmann 1998). The PUM protein is present in germline stem cells at a higher concentration than in the cystoblast; various pum mutations affect either the mitotic ability or the divisional asymmetry of germline stem cells, leading to the depletion of the germline during oogenesis. These observations point to a zygotic role of pum in germline stem cell division.
Images were collected with a Photometrics Star 1 CCD camera and IP Lab software. Images were processed using the Adobe Photoshop and ClarisDraw programs. To quantify the number of pregermline stem cells in the larval ovary, triplicate counting was done to ensure accuracy.

**Nucleic acid manipulations:** Standard molecular biology techniques, such as DNA and RNA preparation, molecular cloning, DNA sequencing, and Southern and Northern blotting, were performed as described in Sambrook et al. (1989).

To define the nature of the *pum* mutations, plasmid rescue was conducted as described in Steller and Pirrotta (1986) to subclone genomic DNA sequences flanking the *P* insertions (Mlodzik and Hirami 1992). Briefly, genomic DNA from *pum* alleles were digested with *Hin*dIII and *Sst*I and ligated with *Sac*I and *Not*I to generate a vector that contains a 2-kb *Hin*dIII fragment and a 1-kb *Sst*I fragment. The digested DNA was ligated into an expression vector designed for the Sunflower matrix (Pierce, Rockford, IL) and used for transformation of *E. coli* cells. The rescue constructs were then used to map the *pum* insertions.

**Germline clonal analysis by FRT-mediated recombination:** The germline requirement of *pum* was determined using the FRT-mediated recombination technique (Stabile and Pirrotta 1986; MacDonald and Spradling 1991). Germline clones were identified by recombination between the FRT sites on the P element and the FRT sites on the rescue plasmid. The presence of the *pum* insertion was verified by Southern analysis using the genomic DNA as a probe. The confirmed plasmids were then used to generate germline clones at the onset of oogenesis.

**Quantitative Western blotting analysis:** Western blot analysis was used to study the pattern of PUM protein expression in *pum* mutants. Ovaries were homogenized in SDS-PAGE sample buffer (25 mm HEPES, pH 7.5, 5 mm MgCl₂, 0.1 mm EDTA, 5 mm β-mercaptoethanol, 1 mm PMSF, 10% glycerol), and insoluble debris was removed by spinning in a microfuge for 10 min at 4°C. The supernatants were heated at 95°C for 10 min and separated on an 8% SDS-polyacrylamide gel (Sambrook et al. 1989). The proteins were then transferred onto a Genescreen membrane (New England Nuclear, Boston, MA) in Tris-glycine buffer (25 mm Tris, 192 mm glycine, pH 8.3). The blots were blocked with 10% nonfat dry milk, 0.3% Tween-20 in PBS. Rat anti-PUM antibodies added at the concentrations PUM2#1 and PUM1637 were used to map the *pum* insertion site. The presence of the *pum* insertion was verified by Southern analysis using the genomic DNA as a probe. The confirmed plasmids were then used to generate germline clones at the onset of oogenesis.

The eclosed females were then crossed to *w; pum*- males and examined for both fertility and ovarian phenotype by Nomarski and immunofluorescence microscopy (see above).

To test whether the germline stem cell clones could be induced in the adult *ovo*D ovaries, we attempted clonal induction in adult females 2–5 days after eclosion. Despite a large number of females (n = 40) induced for the three alleles, none of the *pum-ovo*D strains produced detectable clones (data not shown). This suggests that germline stem cells in these *ovo*D adult females are not mitotically active.

**mRNA in situ hybridization:** To examine the expression pattern of *pum* in the ovary, mRNA in situ hybridization was conducted on ovaries dissected from Oregon-R females, as described in Cox et al. (1998). The probe used in these experiments was a 437-bp PstI DNA fragment from nucleotides 2248–2685 of the *pum* cDNA (see results and Figure 4). This DNA fragment was labeled with digoxigenin using the Genius kit (Boehringer Mannheim, Indianapolis, IN) following the manufacturer's protocols. It was found that altering the length of proteinase K treatment favors the detection of RNA signals in surface cells vs. inner cells (Cox et al. 1998; King and Lin 1999). We therefore used the conditions detailed in Cox et al. (1998) to ensure that all the *pum*-expressing cells were detected.

**RESULTS**

*pum* is required for primordial germ cell development before oogenesis: The *pum* mutations cause failure of germline stem cell maintenance during oogenesis (Lin and Spradling 1997; Forbes and Lehmann 1998). To investigate whether *pum* is also required for germline development before oogenesis, we examined the phenotypes of the ovariole class of *pum* mutants (*pum* alleles) for potential defects in primordial germ cell development. Unlike “classical” maternal effect *pum* mutants that can undergo oogenesis to produce embryos defective in posterior patterning, *pum* mutants show severe oogenic defects and fail to produce any eggs (Lin and Spradling 1997). Third instar larval ovaries from homozygous *pum* mutant and wild-type larvae were stained with anti-VASA antibodies to specifically label germline cells...
**TABLE 1**

<table>
<thead>
<tr>
<th>Allele</th>
<th>Ovaries with the under-proliferated germline&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Ovaries with normal range of germline cells&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Ovaries with the over-proliferated germline&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Total no. of ovaries examined</th>
</tr>
</thead>
<tbody>
<tr>
<td>Canton-S</td>
<td>0 (&lt;i&gt;Na&lt;/i&gt;)</td>
<td>100 (&lt;i&gt;54.6 ± 7.4&lt;/i&gt;)</td>
<td>0 (&lt;i&gt;NA&lt;/i&gt;)</td>
<td>20</td>
</tr>
<tr>
<td>pum&lt;sup&gt;6897&lt;/sup&gt;</td>
<td>54 (&lt;i&gt;19.6 ± 4.4&lt;/i&gt;)</td>
<td>23 (&lt;i&gt;43.5 ± 6.6&lt;/i&gt;)</td>
<td>23 (&lt;i&gt;89.2 ± 9.4&lt;/i&gt;)</td>
<td>13</td>
</tr>
<tr>
<td>pum&lt;sup&gt;2003&lt;/sup&gt;</td>
<td>55 (&lt;i&gt;5.7 ± 2.4&lt;/i&gt;)</td>
<td>45 (&lt;i&gt;66.0 ± 8.1&lt;/i&gt;)</td>
<td>0 (&lt;i&gt;NA&lt;/i&gt;)</td>
<td>11</td>
</tr>
<tr>
<td>pum&lt;sup&gt;4277&lt;/sup&gt;</td>
<td>0 (&lt;i&gt;NA&lt;/i&gt;)</td>
<td>0 (&lt;i&gt;NA&lt;/i&gt;)</td>
<td>100 (&lt;i&gt;123.3 ± 11.1&lt;/i&gt;)</td>
<td>19</td>
</tr>
<tr>
<td>pum&lt;sup&gt;1688&lt;/sup&gt;</td>
<td>0 (&lt;i&gt;NA&lt;/i&gt;)</td>
<td>40 (&lt;i&gt;63.5 ± 7.9&lt;/i&gt;)</td>
<td>60 (&lt;i&gt;132.9 ± 11.5&lt;/i&gt;)</td>
<td>5</td>
</tr>
</tbody>
</table>

The effects of the pum<sup>6897</sup> mutation on germline proliferation, as seen in third instar ovaries, were visualized with anti-VASA immunostaining. Homozygous larvae were selected by lack of the dominant larval <i>Tb</i> marker present on the TM6B chromosome of their heterozygous siblings. Germline cells in each ovary were counted three times, and the results were averaged. The pum<sup>2003</sup> ovaries mostly contain a normal number of germ cells, as reported previously (Lin and Spradling 1997). NA, not applicable.

<sup>a</sup> Ovaries with underproliferated germline contain <40 germ cells (GCs).

<sup>b</sup> Ovaries with normal range of germline cells contain 40-80 germ cells.

<sup>c</sup> Ovaries with overproliferated germline contain >80 germ cells.

(Hay et al. 1990; see materials and methods). Because pregermline stem cells in the third instar ovaries reflect the final stage of primordial germ cell proliferation and migration (see Introduction), any abnormality in number or morphology reflects a defect in primordial germ cell proliferation and development. Wild-type third instar larval ovaries typically contain 54.6 ± 7.4 germ cells located in the medial region of the ovary (Table 1 and Figure 1A). However, ovaries from pum<sup>6897</sup> mutants contain either significantly reduced or increased numbers of germ cells (data from four representative mutants are shown in Table 1). The requirement of pum during primordial germ cell development is further confirmed by dramatic overproliferation of primordial germ cells seen in pum<sup>4277</sup> and pum<sup>1688</sup> mutant ovaries. These observations suggest that pum function is required for the normal proliferation of primordial germ cells before oogenesis.

In addition to abnormal numbers of germ cells, the mutant germ cells also exhibit various morphological defects (Figure 1, B and C). The abnormal size and morphology of pregermline stem cells suggest that the development of primordial germ cells is highly aberrant in pum mutants even though certain germline characteristics, such as VASA expression, are still maintained (Figure 1, B and C). The germline defects are often accompanied by a drastic increase in the size of the ovary, with an increased number of somatic cells (Figure 1, compare B and C to A). These results suggest that pum is also required for the proper formation of the larval ovary.

**pum<sup>6897</sup> mutants are also defective in germline stem cell maintenance, cyst formation and oocyte differentiation during oogenesis:** The proliferation defects of the primordial germ cells and the abnormal morphology of the resulting pregermline stem cells in the pum<sup>6897</sup> larval ovary suggest that these cells may not be able to function normally as germline stem cells during subsequent oogenesis. To test this hypothesis, we examined whether germline defects at the larval stage correlate to subsequent ovarian defects in pupal and adult ovaries.

We first examined the viability of pum<sup>6897</sup> mutants at the pupal stage. The viability difference between the pum<sup>6897</sup> mutants and their heterozygous siblings is within 25% (Figure 1N). This rules out any potentially significant skew in the observed pupal or adult defects caused by selective lethality against pupae with a particular type of oogenic defect.

We then examined the pum<sup>6897</sup> mutant because a high proportion (77%) of pum<sup>6897</sup> larval ovaries contain either underproliferated (54%) or overproliferated (23%) primordial germ cells. Ovaries were isolated from homozygous pum<sup>6897</sup> females at ~48 hr after pupation. They were double stained with anti-VASA antisera to label germ line cells and anti-1B1 antibody to outline somatic cells and label spectrosomes and fusomes, two special structures in the early germline cells (Zaccai and Lipshitz 1996; Deng and Lin 1997). By this stage, pupal ovaries from wild-type siblings have differentiated into an average of 16.7 ± 4.0 ovarioles per ovary (Figure 1D). In mutant ovaries, 1B1 staining of somatic cells shows that a similar number of germaria have also formed (Figure 1E). In contrast to the control ovarioles that always contain a full complement of germline cells, however, 63% of the mutant ovarioles are germlineless (Figure 1F). The remaining mutant ovarioles contain a small number of germline cells. By the adult stage, 86% of the pum<sup>6897</sup> ovaries lack germline (Table 2). These observations indicate that the pum<sup>6897</sup> mutant is severely defective in oogenesis but not in ovarirole formation.

Parallel analyses on six other pum<sup>6897</sup> mutants revealed similar oogenic defects, with the proportion of germlineless ovaries varying among the mutants (Table 2). The remaining ovaries contain either developing egg
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Figure 1.—Phenotype of pum mutants. (A) Wild-type third instar larval ovary stained with an anti-VASA antibody. Approximately 55 pregermline stem cells are located in the medial region of the ovary. (B) pum6897 mutant third instar larval ovary stained for VASA. Germline cells are overproliferated in 23% of the mutant ovaries (see Table 1). However, 54% of the mutant ovaries contain only 19.6 ± 4.4 germ cells. Moreover, these germ cells are grossly abnormal in morphology and size. In addition, the ovary is much larger than the wild-type ovary. (C) pum3203 mutant third instar larval ovary stained for VASA showing underproliferated and defective germline cells. A total of 55% of the pum3203 ovaries contain only 5.7 ± 2.4 germ cells. Specifically, 30% of pum3203 ovaries completely lacked germline cells. (D) A wild-type midpupal ovary stained for VASA; 18 germlia are fully developed and contain a full complement of germ cells. (E and F) A pum6897 midpupal ovary stained with anti-1B1 and anti-VASA antibodies, respectively. (E) Although individual germaria have formed, (F) many of them do not contain germline cells, as marked by arrows. (G) Wild-type adult ovarioles stained with DAPI showing germlia (g) that have produced a string of developing egg chambers. (H) A VASA-stained pum1688 ovariole containing only two mature egg chambers and a rudimentary germarium (g). (I) A VASA-stained pum4277 ovariole containing three clusters of undifferentiated germ cells, presumably because of the loss of asymmetry during the division of three germline stem cells.

Figure 1H illustrates that the oogonia produced by these mutant egg chambers are affected by the pum mutation. In the wild-type, the developing egg chamber produces a string of three mature egg chambers (Figure 1H), each containing a full complement of nurse cells and oocytes. In the pum mutants, however, the egg chamber shows a reduced number of nurse cells and no oocytes (Figure 1, J–L). This defect also exists in the maternal effect lethal class of pum mutants. Staining of these mutant egg chambers with rhodamine-conjugated phalloidin reveals a reduced number of ring canals (Figure 1J), indicating that the reduced number of nurse cells results from reduced divisions of cystoblasts. Furthermore, these mutant egg chambers show a pronounced unequal ploidy events.

chambers (Figure 1H) or undifferentiated germ cell clusters (Figure 1I), as described previously for the pum2003 mutation (Lin and Spradling 1997). This confirms the previous conclusion that the self-renewing asymmetric division of germline stem cells is disrupted in the pum2003 mutant.

pum mutations also affect subsequent oogenic events. Developing mutant egg chambers sometimes contain few nurse cells but no oocytes (Figure 1, J–L). This defect also exists in the maternal effect lethal class of pum mutants. Staining of these mutant egg chambers with rhodamine-conjugated phalloidin reveals a reduced number of ring canals (Figure 1J), indicating that the reduced number of nurse cells results from reduced divisions of cystoblasts. Furthermore, these mutant egg chambers show a pronounced unequal ploidy...
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functions cell autonomously in the germ cell clusters of pum

pumET1

from germ cells at 2, B and C). Sometimes, differentiated ovarioles contain

pum4277

malignant blocks oogenesis in a cell-autonomous manner, arresting egg chambers uniformly at stage 3 (Figure 2; for staging see King 1970). This defect is distinctively different from either the germlineless or differentiating phenotype of pum (see above) or the wild-type ovariole. The developmental fate of homozygous pum germ-line stem cell clones depends on whether pum is required during oogenesis. If pum is required during oogenesis, pum- clones should show corresponding pum-oogenic defects. Alternatively, if pum is not required during oogenesis, the pum- clones should undergo oogenesis normally.

For all three pum alleles tested, pum- germ-line clones exhibited typical pum- defects. For example, many pum germ-line clones produced ovarioles that contain only one to three mature egg chambers but no other germ cells (Figure 2A). These typical differentiated pum germ-line ovarioles are distinctively different from the ovarioles (Figure 2A). The differentiated ovarioles are also seen in pum and pum germ-line clones (Figure 2, B and C). Sometimes, differentiated ovarioles contain four to five egg chambers (Figure 2D), indicating that these pum- germ-line stem cells have divided once before entering oogenesis.

A quantitative summary of the clonal analysis is presented in Table 3. In addition, pum, pum, and pum clonal ovarioles contained an average of 4.2 ± 2.0 (n = 17), 2.8 ± 1.7 (n = 21) and 2.2 ± 1.5 (n = 14) egg chambers, respectively. Other defects, such as germlineless germaria (see Figure 1, E and F) and ovarioles with undifferentiated germ cell clusters (see Figure 1L), though more difficult to quantify, also exist in the clone-induced ovarioles. These defects indicate that pum activity is directly required in the germ-line stem cells during oogenesis.

### Table 2

Germlineless vs. differentiated ovary phenotypes of pum ovaries

<table>
<thead>
<tr>
<th>Allele</th>
<th>Germlineless ovaries (%)</th>
<th>Differentiated ovaries (%)</th>
<th>Mixed ovaries (%)</th>
<th>No. of ovaries examined</th>
</tr>
</thead>
<tbody>
<tr>
<td>pum6937</td>
<td>86</td>
<td>7</td>
<td>7</td>
<td>72</td>
</tr>
<tr>
<td>pum3203</td>
<td>31</td>
<td>31</td>
<td>38</td>
<td>64</td>
</tr>
<tr>
<td>pum4777</td>
<td>0</td>
<td>89</td>
<td>11</td>
<td>9</td>
</tr>
<tr>
<td>pum4688</td>
<td>9.5</td>
<td>62</td>
<td>28.5</td>
<td>21</td>
</tr>
<tr>
<td>pum4006</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>pum7088</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>12</td>
</tr>
<tr>
<td>pum2003</td>
<td>43</td>
<td>32</td>
<td>25</td>
<td>28</td>
</tr>
</tbody>
</table>

* Ovaries completely depleted of germline cells, as indicated by the absence of VASA expression. All flies were dissected at 3 days posteclosion in this experiment.

* Ovaries that contain recognizable egg chambers and/or germline cells in at least one ovariole. These ovaries frequently contain only one or a few ovarioles with egg chambers and/or germ cells. For example, although 32% of the pum ovaries are classified as differentiated ovaries, most of these ovaries contain only one or a few ovarioles with egg chambers. Thus, the percentage of ovaries with egg chambers is similar to the 2% value reported previously (Lin and Spradling 1997). A similar phenomenon exists for the “mixed ovaries” (see below).

* Sets of contralateral ovary pairs that had one germlineless and one differentiated ovary.

among these nurse cells (Figure 1L), indicating that the endoreplication mechanism in these egg chambers has been affected severely. These defects suggest that pum is required for the proper division of cystoblasts and differentiation of germline cysts.

In addition to germline defects, somatic defects were also detected at a low frequency in pum mutants, such as long interfollicular stalks (Figure 1K) and the disruption of single-stack cells in the terminal filament (Figure 1M). Together, these data reveal that multiple germline and somatic processes of oogenesis are disrupted by pum mutations.

**pum is directly involved in germ-line stem cell division during oogenesis:** The severe defects observed in pum mutant primordial germ cells suggest that these cells are unlikely to undergo normal oogenesis, which precludes the opportunity to analyze whether pum also plays a direct role in germ-line stem cell division and other oogenic processes. To overcome this problem, we let germ cells develop normally before oogenesis and then removed the pum activity in germ-line stem cells at the onset of oogenesis by inducing homozygous pum germ-line clones using the FLP-DFS technique (Chou and Perrimon 1992; see materials and methods). Forbes and Lehmann (1998) have shown by pole cell transplantation that pum functions cell autonomously in the germ-line. Thus, by removing pum from germ cells at the onset of oogenesis, we were able to examine directly whether pum is required during oogenesis.

We induced the homozygous pum mutant germ-line in pum, pum, and a maternal effect mutation pum, because they are strong mutations representing three types of molecular lesions (see below). Homozygous pum germ-line clones were generated in the ovo background (see materials and methods). ovo dominantly blocks oogenesis in a cell-autonomous manner, arresting egg chambers uniformly at stage 3 (Figure 2; for staging see King 1970). This defect is distinctively different from either the germlineless or differentiating phenotype of pum (see above) or the wild-type ovariole. The developmental fate of homozygous pum germ-line stem cell clones depends on whether pum is required during oogenesis. If pum is required during oogenesis, pum- clones should show corresponding pum- oogenic defects. Alternatively, if pum is not required during oogenesis, the pum- clones should undergo oogenesis normally.

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A quantitative summary of the clonal analysis is presented in Table 3. In addition, pum, pum, and pum clonal ovarioles contained an average of 4.2 ± 2.0 (n = 17), 2.8 ± 1.7 (n = 21) and 2.2 ± 1.5 (n = 14) egg chambers, respectively. Other defects, such as germlineless germaria (see Figure 1, E and F) and ovarioles with undifferentiated germ cell clusters (see Figure 1L), though more difficult to quantify, also exist in the clone-induced ovarioles. These defects indicate that pum activity is directly required in the germ-line stem cells during oogenesis.
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Figure 2.—Oogenetic defects of pum germline clones induced at the third instar larval stage. All the ovaries were dissected at 9–12 days after eclosion and were stained with anti-1B1 antibodies to outline ovarian cells. (A) A pair of pum2003 ovaries. The right ovary contains only arrested ovoD1 ovarioles (ovoD1), while the left ovary contains multiple homozygous pum2003 clonal ovarioles (three ovarioles are shown and labeled as pum2003). Each pum2003 clonal ovariole contains two to three mature eggs, but no other germ cells. This is in sharp contrast to ovoD1 ovarioles, which contain numerous egg chambers arrested at stage 3. (B) Several pum1688 clonal ovarioles. One ovariole contains a single mature egg and a rudimentary germarium. (C) A pumET1 clonal ovariole (pumET1) embedded in a pair of ovoD1 ovaries. (D) A pum1688 clonal ovary in a 12-day-old female containing five egg chambers, suggesting some residual germline stem cell activity in this ovariole.

pum appears to be required in somatic cells for oviposition: A striking difference between the pum germline clonal females and their corresponding nonclonal homozygous females is that the eggs produced by the clonal females are often laid, yet eggs produced by their nonclonal counterparts are never laid. This suggests that pum may be required in somatic cells for oviposition. To further test this possibility, we let the germline clonal females lay eggs for 9 days and then dissected the females to count the number of eggs that were still held in the ovary to measure the efficiency of oviposition. Among three alleles tested, pum2003, pum1688, and pumET1 clonal females achieved oviposition efficiency of 56, 85, and 78%, respectively (for absolute number of eggs laid by the females, see Table 3). This egg-laying ability is never observed in homozygous pum2003 or pum1688 females. The restoration of the egg-laying ability in females whose soma is no longer deficient in pum suggests that the pum gene is required in somatic cells for oviposition. This function is affected by pum2003 and pum1688 mutations as well as by the maternal effect class of mutations such as pumET1.

pum2003 and pum1688 mutations do not abolish the maternal effect function of pum in embryos: A more surprising observation is that, despite pum2003 or pum1688 mutations causing both germline and somatic defects in

### TABLE 3

Germline clonal analysis of *pumilio*

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Clonal induction</th>
<th>Total no. females examined</th>
<th>No. of clone-containing females</th>
<th>No. of females laying eggs</th>
<th>No. of eggs laid/egg-laying female</th>
<th>Viable progeny/females</th>
</tr>
</thead>
<tbody>
<tr>
<td>FRT ovo1Δ/pum2003</td>
<td>+</td>
<td>23</td>
<td>10</td>
<td>10</td>
<td>40.4 ± 6.3</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>–</td>
<td>16</td>
<td>0</td>
<td>0</td>
<td>N/A</td>
<td>0</td>
</tr>
<tr>
<td>FRT ovo1Δ/pum1688</td>
<td>+</td>
<td>16</td>
<td>7</td>
<td>6</td>
<td>36 ± 6.0</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>–</td>
<td>22</td>
<td>0</td>
<td>0</td>
<td>N/A</td>
<td>0</td>
</tr>
<tr>
<td>FRT ovo1Δ/pumET1</td>
<td>+</td>
<td>16</td>
<td>10</td>
<td>3</td>
<td>4.6 ± 2.1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>–</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>N/A</td>
<td>0</td>
</tr>
</tbody>
</table>
ogenetic and opositional (see above), eggs laid by females containing the homozygous pum^1688 or pum^1203 germline were sometimes capable of developing into adulthood (Table 3). This is in contrast to eggs laid by females containing the homozygous pum^ET1 germline, which showed typical posterior patterning defects and failed to hatch, as reported previously for maternal effect alleles (Lehmann and Nüsslein-Volhard 1987).

The ability of the pum^1203 or pum^1688 eggs to develop further suggests that pum^1203 or pum^1688 mutations do not abolish the maternally provided pum activity required for embryogenesis. The pum^- embryonic eggs were produced by mating virgin mutant pum^1203 or pum^1688 clonal females to homozygous pum^1203 or pum^1688 males, respectively. This rules out any possible paternal contribution to the embryonic pum function.

pum^1688 partially complements other pum^et and maternal effect pum alleles: Both pum^1203 and pum^1688 mutations lead to more defects than maternal effect mutations during oogenesis, yet they do not completely affect embryonic development. Conversely, maternal effect mutations have fewer pleiotropic effects during oogenesis, yet they completely block embryogenesis. These differences could suggest that maternal effect mutations are stronger mutations. Alternatively, it is possible that pum is a complex locus encoding several discrete and complementary functions. To test these possibilities, we conducted inter se genetic complementation tests among pum^et and maternal effect alleles of pum. Previous complementation analyses had shown that pum^1203 and pum^1688 failed to complement several of the maternal effect lethal pum alleles, yet pum^1688 partially complements the maternal effect alleles (Lin and Spradling 1997). We further quantified the degree of complementation (Figure 3I). The results reveal that pum^1688 partially complements the embryonic lethality of maternal effect alleles pum^ET1, pum^MC, and pum^FTT; of these, pum^MC and pum^FTT are known to disrupt the RNA-binding domain at the C terminus of the PUM protein (Forbes and Lehmann 1998). This complementation leads to the production of viable yet sterile progeny. In addition, pum^1688 also partially complements pum^1203 and pum^1203 of the pum^et class for oogenic defects and embryonic lethality (Figure 3, E and I). However, trans-heterozygotes between pum^1688 and all maternal effect alleles tested, despite partial complementation for lethality, showed oogenic defects (Figure 3, B-D) similar to those of the pum^et (Figure 3E) or maternal effect class mutants (Figure 3, G and H), but not to those of the wild-type ovary (Figure 3A). For example, ovaries from the trans-heterozygous females are often depleted of germline (Figure 3B, compare to Figures 1, E and F, and 3H) or contain differentiated ovarioles with only a few mature eggs (Figure 3C, compare to Figures 1H and 3G). These data indicate that germline stem cell division defects fail to be complemented in these heteroallelic combinations, even though the oviposition defect of pum^1688 has been complemented by the maternal effect alleles and the embryonic lethality of the maternal effect alleles has been complemented by the pum^1688 allele.

pum^et mutations map within the pum transcription unit: To study the molecular nature of the pum^et mutations, we isolated genomic DNA sequences encompassing the PZ-element insertion sites in the pum^et mutants (see materials and methods). Molecular mapping of these genomic clones with respect to the cosmids clones spanning the pum locus (Barker et al. 1992; MacDonald 1992) revealed that all the pum^et PZ insertions are located in introns of the pum gene (Figure 4). Among them, the pum^1688 insertion is located between exons 3 and 4 while other pum^et insertions are clustered within a 505-bp region at the center of a 120-kb intron between exons 8 and 9. All the PZ insertions are oriented in the same direction so that the rosy gene in the PZ element is toward the 5′ side of the pum gene while the lacZ gene is oriented toward the 3′ side. This orientation of PZ insertions in introns has been shown by Horowitz and Berg (1995) to cause aberrant splicing and transcription termination because of the presence of a splice acceptor site in the l(3)s12 sequence near the rosy gene (Figure 4). Consistent with this, we identified a new P-insertional allele, p(3)1196, carrying a P element lacking the l(3)s12 sequence (Rorth 1996). Even though p(3)1196 is inserted in the same orientation and location, 185 base pairs 5′ to pum^1688, it does not cause any detectable lethality or sterility.

pum^1203 and pum^1688 eliminate two different PUM proteins, each of which is sufficient for maternal effect function, yet both of which are required for oogenesis: The partial complementation between different P alleles suggests that they may differentially affect the pum gene product. Although two splicing variants of the pum transcript have been characterized (Barker et al. 1992), they differ only in the 5′-untranslated region but share the same predicted ORF (Figure 4). However, previous analysis of the PUM protein from embryonic extracts detected two bands positioned at ~156 kD among several proteins with smaller molecular weight (MacDonald 1992; Murata and Wharton 1995). It was known only that the 156-kD doublet represents the PUM protein. Because the pum^1203 P insertions were located within the pum transcription unit, we performed Western blot analysis to examine how these mutations affect the expression of different bands that are recognized by anti-PUM antibodies. Because pum^1688 complements other pum^et alleles and potentially causes aberrant splicing at a different exon than other pum^et alleles, we tested how pum^1688 and other pum^et alleles affect the expression of PUM isoforms, using pum^1203 and pum^4277 as representatives of other pum^et alleles.

Western blots of Drosophila ovarian extracts prepared from flies homozygous for pum^1688, pum^1203, and pum^4277 mutations were probed with four different antibodies made against the PUM protein (MacDonald 1992;
Figure 3.—Phenotypes of pum heteroallelic combinations. (A) Anti-VASA stained pum<sup>1688</sup>/TM3 ovaries show wild-type morphology. (B) A pair of DAPI-stained pum<sup>1688</sup>/pum<sup>ET7</sup> ovaries display a typical germfree morphology (compare to Figure 1E and F). (C) A pair of anti-1B1-stained pum<sup>1688</sup>/pum<sup>ET7</sup> ovaries exhibit normal differentiated phenotype, with the few egg chambers in each ovariole developed to maturity (compare to Figure 1H). (D) DAPI-stained pum<sup>1688</sup>/pum<sup>MSC</sup> ovary also showing differentiated phenotype. (E) DAPI-stained pum<sup>1688</sup>/pum<sup>3203</sup> ovary displaying a mildly differentiated phenotype. (F) A pair of DAPI-stained pum<sup>3203</sup>/pum<sup>MSC</sup> ovaries containing both germfree and differentiated ovarioles. (G) Anti-VASA stained pair of differentiated pum<sup>MSC</sup>/pum<sup>ET9</sup> ovaries. (H) DAPI-stained pair of germfree pum<sup>MSC</sup>/pum<sup>ET9</sup> ovaries. (I) Histogram showing the number of progeny from heteroallelic combinations normalized to wild-type controls.
**Figure 4.** Location of the pum mutations in the pum locus. The map of pum is adapted from Barker et al. (1992), with transcription proceeding from right to left corresponding to a distal-to-proximal orientation on chromosome 3. Exons are numbered serially from 5' to 3'. Open-boxed exons denote the 5'-untranslated region in the mRNA, while the black exons encode the ORF. Each of the P-insertion sites is marked by a triangular flag, with the direction of the flag pointing to the rosy (ry) side of the P element. The P element in all the alleles, except for that in ep(3)1196, is the PZ element, whose structure is shown in the lower part of the figure at a different scale. A left-pointing arrow in the l(3)S12 region (shaded area) of the PZ element indicates the splice acceptor site.

Note that the pum1688 P insertion is located in intron sequence upstream of exon 4, ~60 kb distal to the other puminsertions, which are clustered within a 505-bp region at the center of a 120-kb intron between exons 8 and 9. Among them, the 3'-most alleles, pum670 and pum7098, are 387 bp from pum2003 and have identical insertion sites despite being independently isolated lines. The pum2003 and pum3906 insertions are separated by a single base pair. pum3203 and pum4277 are located at more 5' sites, 109 and 118 bp from pum2003 and pum4277, respectively. The pum RNA probe used for in situ hybridization is derived from exons 4–6 and the beginning of exon 7. The 15-amino-acid peptide used for generating the Ovtpep2 antibody corresponds to a sequence in exon 4.

Forbes and Lehmann 1998; this study, see materials and methods). Protein prepared from pum1293/pumET9 and pum1293/pumET1 transheterozygotes, all of which have been proposed to be null mutations (Forbes and Lehmann 1998), were used as negative controls. All antisera tested showed two major bands, the 156- and the 130-kD bands, in wild-type flies (Figure 5). The 156-kD band can be resolved as a doublet upon shorter exposure. This concurs with previous Western blotting analyses (MacDonald 1992). Interestingly, the pum1688 mutation completely eliminates the 156-kD doublet but does not affect the 130-kD band. Conversely, pum2003 and pum4277 mutations diminish the 130-kD band but do not appear to affect the 156-kD doublet (Figure 5). These results suggest that both the 156-kD doublet and the 130-kD band are functionally important protein isoforms of the pum gene. Affecting either one of the isoforms leads to defective pum function during preoogenic germline development and oogenesis, but one of the isoforms is sufficient for embryogenesis (see above). The heteroallelic pum1293/pumET9 and pum1293/pumET1 combinations eliminated both the 156-kD doublet and the 130-kD isoform (for pum1293/pumET9, see Figure 5C), suggesting that these three maternal effect mutations are null mutations.

A novel band of ~88 kD appears in the homozygous pum2003 and pum4277mutants, but not in pum1688, in maternal effect pum mutants, or in the wild-type flies (see Figure 5; not shown are pum4277 data, which are identical to those of pum2003). Thus, this band is likely to be the product of aberrant splicing specific to pum2003, pum4277,
and other pum<sup>91</sup> mutations residing in the 120-kb intron. This 88-kD band was detected by antibodies against the C-terminal region of the PUM protein containing the RNA-binding domain downstream of all the pum<sup>96</sup> insertion sites, suggesting that the aberrant splicing does not cause premature termination of protein synthesis at the pum<sup>96</sup> insertion sites, as reported previously (Horowitz and Berg 1995). Instead, it suggests a novel splicing activity of the PZ-element insertions that awaits further investigation.

In addition to the 156-kD doublet and the 130-kD isoform, several other weak bands of lower molecular weights are also detected by the anti-PUM antibodies in wild-type flies. Among them, a weak band of ~98 kD is reduced in both pum<sup>1688</sup> and pum<sup>2003</sup> mutants (Figure 5), suggesting its possible involvement in pum function. Other bands, however, are not altered or eliminated in the pum mutants and, thus, are unlikely to be pum products.

A pum cDNA transgene containing the known ORF encodes only the known 156-kD PUM protein and rescues the pum<sup>96</sup> preoogenic and oogenic defects: The 156- and 130-kD isoforms of PUM could both derive from the known ORF of pum by posttranslational processing (Figure 4). Alternatively, one of them could be encoded by a novel species of alternatively spliced pum mRNA that is yet to be identified. To discriminate between these possibilities, we introduced into pum<sup>1688</sup> and pum<sup>2003</sup> mutants a P[nos-pum] transgene that contains a pum cDNA encoding the known ORF driven by the nanos promoter (Barker et al. 1992). These mutant backgrounds allowed us to determine which PUM isoform this cDNA encodes and which pum mutant defect it can rescue (see materials and methods). This test thus also served the purpose of allowing us to examine which PUM isoform is responsible for a particular developmental process. Western analysis revealed that the missing 156-kD isoform in the homozygous pum<sup>1688</sup> mutant is clearly replenished by the P[nos-pum] transgene, while the missing 130-kD isoform in the homozygous pum<sup>2003</sup> mutant is not restored (Figure 6A). These results indicate that the known pum ORF encodes only the 156-kD isoform.

Although the P[nos-pum] transgene encodes only the 156-kD isoform, it rescues the preoogenic and oogenic defects of both pum<sup>2003</sup> and pum<sup>1688</sup> mutants. In both homozygous pum<sup>2003</sup> and pum<sup>1688</sup> mutants carrying the transgene, most ovarioles contain actively dividing germline stem cells that support normal oogenesis, even in 8-day-old mutant females, as is evident by the presence of a progression of wild-type egg chambers in most of their ovarioles (Figure 6, B–D). This indicates the complete rescue of germline and oogenic defects of both pum<sup>2003</sup> and pum<sup>1688</sup> mutants by the P[nos-pum] transgene. Thus, even though the lack of either the 156- or 130-kD isoform leads to severe defects during zygotic germline development and oogenesis, increasing the expression of the 156-kD isoform alone can compensate for the lack of the 130-kD isoform and rescue the germline and oogenic defects.

In addition to the complete oogenic rescue, 8 out of 15 transgene-carrying pum<sup>2003</sup> females produced progeny; 3 out of 11 transgene-carrying pum<sup>1688</sup> females also produced progeny (Figure 6E). These observations further support the conclusion that the 156- and the 130-kD PUM isoforms are not functionally distinct. Either isoform is sufficient for the maternal effect function of pum (see above).

Despite the rescue of the female sterility, P[nos-pum] does not rescue the semilethality of pum<sup>2003</sup>. The viability of homozygous P[nos-pum]; pum<sup>2003</sup>/pum<sup>2003</sup> flies is 14% of that of P[nos-pum]; pum<sup>2003</sup>/ + flies, which is similar to the viability of pum<sup>2003</sup>/pum<sup>2003</sup> flies (Lin and Spradling 1997). Thus, as expected, the somatic function of pum in supporting viability is not rescued by the germline-specific expression of the P[nos-pum] transgene.

pum is expressed in the soma and germline in the ovaries: Phenotypic analysis reveals a somatic function for pum during oogenesis, yet previous RNA and protein in situ analyses showed only the germline expression of pum (MacDonald 1992; Forbes and Lehmann 1998). To detect the possible somatic expression of pum, we first examined the enhancer trap staining pattern of most pum<sup>96</sup> mutations (see materials and methods), which revealed that they are specifically expressed in the terminal filament cells (Figure 7A), as was reported previously for pum<sup>2003</sup> (Lin and Spradling 1997). To test whether this reflects part of the pum expression pattern, we conducted RNA in situ hybridization to whole-mount wild-type ovaries, using conditions that allow the detection of RNA in both surface somatic cells and inner germline cells (Cox et al. 1998; also see materials and methods). Under conditions that favor the detection in surface cells, pum RNA is easily detectable in the terminal filament cells and epithelial sheath cells in the interfollicular stack region but is barely detectable in the follicle cells (Figure 7B). In the germline, pum mRNA is present in the germarium and later stages of oogenesis, as reported previously (MacDonald 1992). The terminal filament and interfollicular cell expression may reflect the involvement of pum in ovary differentiation and oviposition (see discussion).

Immunostaining using different anti-PUM antibodies consistently revealed that the PUM protein is present in several somatic and germline cell types in the ovaries, including the terminal filament and the invaginating follicle cells in germarial regions IIb and III, as well as postgermarial follicle cells (Figure 7C). In the germline, PUM is present at the highest level in germline stem cells and at lower levels in other germarial germline cells, as described previously (Forbes and Lehmann 1998). The follicle cell expression of PUM may be related to the aging property of follicle cells observed by Forbes and Lehmann (1998).
DISCUSSION

The pum gene has been shown to be required for embryonic patterning and germline stem cell division during oogenesis (Nüsslein-Volhard et al. 1987; Lin and Spradling 1997; Forbes and Lehmann 1998). It is known to encode a 156-kD protein (MacDonald 1992; Murata and Wharton 1995). In this study, we have reported the novel function of pum in primordial germ cell proliferation, ovary formation, oogenesis, and oviposition. Moreover, we show that pum encodes two major functional PUM isoforms that are required in various developmental processes.

**pum is involved in primordial germ cell proliferation, ovary formation, oogenesis and oviposition:** Although a number of genes involved in germ cell formation and migration have been identified (reviewed in Wilson and MacDonald 1993; also see Warrior 1994; Nakamura et al. 1996; Zhang et al. 1996; Moore et al. 1998), few genes, if any, are known to affect the proliferation and development of primordial germ cells in the embryonic-larval gonad during preoogenic development. pum is required for primordial germ cell development because its mutant larval ovaries contain germ cells that are aberrant both in number and morphology. In pum<sup>mt</sup> mutants that possess partial pum activity, both under- and overproliferation of primordial germ cells were observed. These contrasting defects are not only seen in pum<sup>mt</sup> alleles with similar molecular lesions, but also in the same mutant. This suggests that pum does not simply promote or suppress the mitotic ability of primordial germ cells. Moreover, pum<sup>mt</sup> mutant primordial germ cells also vary greatly in size. This suggests that pum is involved in primordial germ cell proliferation, ovary formation, oogenesis and oviposition. Moreover, we show that pum encodes two major functional PUM isoforms that are required in various developmental processes.

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In addition to its role in primordial germ cell develop-
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During oogenesis, and oviposition after oogenesis. The diverse functions of pum in the germline and soma again suggest that it may regulate the translation of distinct sets of mRNAs in different types of cells. Consistent with this, pum is expressed in the germline, as well as in the terminal filament and interfollicular epithelial sheath cells. The terminal filament expression may reflect a role for pum in ovariole formation during overydifferentiation. This role appears to be redundant, because ovariole formation is largely unaffected in pum mutants. The epithelial sheath expression may facilitate oviposition, because the rhythmic contraction of the sheath around the egg chambers is believed to squeeze egg chambers into the oviduct (Mahowald and Kambsellis 1980). In fact, the somatic function of pum is apparently not limited to the ovary, because pum-mutants are semilethal (Lehmann and Nüsslein-Volhard 1987; Lin and Spradling 1997; this study). Hence, pum may mediate a translational regulation mechanism that is broadly used in many developmental processes. Although hunchback mRNA, which encodes a zinc finger transcriptional factor, has been identified as a direct downstream target of pum during embryonic patterning (Murata and Wharton 1995; Zamore et al. 1997), this regulatory relationship does not appear to exist during oogenesis (M. Parisi and H. Lin, unpublished data). The identification of novel target genes of pum in different developmental processes should provide exciting opportunities to study the role of translational repression during development.

**pum is directly required for germline stem cell division:** Previous studies have suggested an essential role for pum in germline stem cell maintenance during oogenesis (Lin and Spradling 1997; Forbes and Lehmann 1998). However, it was not known whether pum is directly involved in this process or if the germline stem cell defect is the result of an earlier requirement of pum in germ cell development. In this study, we have generated pum- clones in germline stem cells at the onset of oogenesis. This experiment allowed us to demonstrate the direct involvement of pum in germline stem cell division. In pum mutants, some ovarioles contain two to three egg chambers but lack other germ cells, suggesting that the two to three germline stem cells differentiate without self-renewing divisions. On the other hand, the existence of undifferentiated germ cell clusters suggests that pum is also required for the differentiation or asymmetry of germline stem cell division (Lin and Spradling 1997; this study). pum may achieve these two functions by mediating differential translation of its target mRNAs in the two daughter cells. As a result, one daughter cell will contain more mitotic factors and fewer differentiation factors and, thus, remain a stem cell, while the other daughter cell will contain more differentiation factors and fewer mitotic factors and, thus, differentiate into a cystoblast. In pum mutants, the translation of the pum target genes occurs development during oogenesis, and oviposition after oogenesis.
evenly or stochastically in the two daughter cells. This would create the stochastic fluctuation of mitotic and differentiation factors in the daughter cells, leading to the random choice of their cell fate to differentiate vs. to divide without differentiation. As a result, germline stem cells are not maintained in any of the pum mutant ovarioles.

This differential translation model is consistent with the differential distribution of PUM proteins between germline stem cells and cystoblasts (Forbes and Lehmann 1998; this study). At the present time, it is not known whether the presence of a high level of PUM in the stem cells vs. a low level of PUM in cystoblasts can lead to the differential translational mechanism proposed above. Also, it is not known what controls the differential distribution of PUM in the two daughter cells. Germline stem cells are always in direct contact with the terminal filament while cystoblasts are always one cell away from the terminal filament (Deng and Lin 1997; Lin and Spradling 1997). Given the important role of the terminal filament in the self-renewal of germline stem cells (Cox et al. 1998; King and Lin 1999), it is likely that this somatic signaling controls the asymmetric activity of PUM in the two daughter cells. In the future, analyzing the asymmetric PUM activity in the two daughter cells and how it is regulated by cell-cell signaling should significantly advance our understanding of the self-renewing mechanism of stem cells.

The 130-kD protein is a functional product of the pum gene: Although the known ORF of pum predicts a 156-kD full-length protein (Barter et al. 1992; Macdonald 1992), our analysis reveals that the 130-kD protein is either translated from an alternatively spliced version of the pum RNA transcript containing the first 130 kD protein (Dent and Lin 1997; Lin and Spradling 1997). Despite the lack of knowledge about the exact structural relationship between the 156- and 130-kD isoforms, our analysis clearly indicates that they are both involved in pum function in vivo. The pum1688 mutant and other pumNT mutations share very similar germline defects in primordial germ cell development and larval ovary formation, suggesting that both the 156- and 130-kD isoforms are involved in these preoogenic processes. Our germline clonal analyses of pum1688, pum1203, and pumNT further suggest that pum is continuously required in germline stem cell division and germline cyst formation during oogenesis. Moreover, these analyses also suggest that both the 156- and the 130-kD isoforms continue to contribute to similar functions in these oogenic processes, because removing either isoform in the germline during oogenesis causes similar defects, yet removing both isoforms causes the most severe defect (see results). Finally, pum1688 and pum1203 mutants also share very similar defects in larval ovary formation and oviposition. These results indicate that the 156- and 130-kD isoforms share very similar developmental functions.

The functional similarity of the 156- and 130-kD isoforms is further illustrated by the complete rescue of the preoogenic and oogenic germline defects of pum1688 and pum1203 by the P[nos-pum] transgene, which only
expresses the 156-kD protein (Figure 6). It seems that not only replenishing the missing 156-kD isoform in the pum^{168} mutant can restore oogenesis, but increasing the level of the 156-kD isoform in the pum^{1003} mutant can also compensate for its deficiency in the 130-kD isoform to reinitiate oogenesis. Thus, the 156- and 130-kD isoforms can compensate for each other's function in a dosage-dependent manner.

Either the 156- or 130-kD isoform is sufficient for the maternal effect function of pum during embryogenesis: The fact that the homozygous pum^{168} and pum^{1003} germlines, but not the pum^{1017} germlines, can produce embryos capable of developing into adulthood indicates that either the 156- or the 130-kD isoform is sufficient for the maternal effect function of pum during embryogenesis. An interesting conclusion one can derive from these observations is that the zygotic function of pum in preoogenic germline development and oogenesis may require a higher dose of pum expression than the maternal effect function during embryogenesis. Alternatively, the maternal loading of both 156- and 130-kD PUM isoforms into the embryo may be abundant so that either isoform alone can provide a sufficient dose of PUM activity to support embryogenesis. Finally, the difference between the zygotic and maternal effects may result from the combination of both mechanisms.

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