Development 125, 679-690 (1998)
Printed in Great Britain © The Company of Biologists Limited 1998
DEV3776

Nanos and Pumilio have critical roles in the development and function of *Drosophila* germline stem cells

Alexandria Forbes and Ruth Lehmann*

Howard Hughes Medical Institute and Skirball Institute, NYU Medical Center, 540 1st Avenue, New York, NY 10016, USA
*Author for correspondence (e-mail: lehmann@saturn.med.nyu.edu)

Accepted 25 November 1997: published on WWW 22 January 1998

SUMMARY

The zinc-finger protein Nanos and the RNA-binding protein Pumilio act together to repress the translation of maternal *hunchback* RNA in the posterior of the *Drosophila* embryo, thereby allowing abdomen formation. *nanos* RNA is localized to the posterior pole during oogenesis and the posteriorly synthesized Nanos protein is sequestered into the germ cells as they form in the embryo. This maternally provided Nanos protein is present in germ cells throughout embryogenesis. Here we show that maternally deposited Nanos protein is essential for germ cell migration. Lack of zygotic activity of *nanos* and *pumilio* has a dramatic effect on germline development of homozygous females. Given the coordinate function of *nanos* and *pumilio* in embryonic patterning, we analyzed the role of these genes in oogenesis. We find that both genes act in the germline. Although the *nanos* and *pumilio* ovarian phenotypes have similarities and both genes ultimately affect germline stem cell development, the focus of these phenotypes appears to be different. While *pumilio* mutant ovaries fail to maintain stem cells and all germline cells differentiate into egg chambers, the focus of *nanos* function seems to lie in the differentiation of the stem cell progeny, the cystoblast. Consistent with the model that *nanos* and *pumilio* have different phenotypic foci during oogenesis, we detect high levels of Pumilio protein in the germline stem cells and high levels of Nanos in the dividing cystoblasts. We therefore suggest that, in contrast to embryonic patterning, Nanos and Pumilio may interact with different partners in the germline.

Key words: Nanos, Pumilio, Germ cell, Migration, Germline, Stem cell, Oogenesis, *Drosophila*

INTRODUCTION

In many organisms, primordial germ cells are set aside from the cells that give rise to the soma early in development. Subsequently, germ cells migrate through the developing embryo to associate with the cells that will form the somatic portion of the gonad. There, a population of germline stem cells is established which will continue to supply the organism with the cells that differentiate into sperm or egg throughout adult life. Although the progress of germline cells from their formation in the early embryo to the production of eggs and sperm has now been described in a number of organisms, little is known about the mechanisms underlying the specification of the germline, its migration through the embryo and the establishment and maintenance of the germline stem cells. In this paper, we show that two maternal effect genes, *nanos* (*nos*) and *pumilio* (*pum*), in addition to determining the pattern in the soma of the embryo, also play an important role in germline development and function.

In *Drosophila*, the germ cells are formed at the posterior pole of the embryo before the rest of the embryo cellularizes. During gastrulation, the 30-40 germ cells are carried inward in the primordium of the posterior midgut. They then move through the gut and migrate dorsally over the basal surface of the gut before transferring to the overlying mesoderm (Fig. 2A). Here the germ cells split into two bilateral groups that move laterally to associate with the precursors of the somatic portion of the gonad in parasegments 10-12 (Fig. 2C). These somatic and germline cells then coalesce to form an embryonic gonad (reviewed in Williamson and Lehmann, 1996).

In female flies, the somatic and germline component of the gonad proliferate during larval life and ovary morphogenesis begins in late 3rd instar larvae (Godt and Laski, 1995). The adult ovary contains 14-16 ovarioles each with a germarium at the tip, within which the germline and somatic stem cells give rise to the cells that assemble into egg chambers. The rest of the ovariole is made up of a chain of progressively developing egg chambers (King, 1970). Two or three germline stem cells are located at the tip of the germarium in close proximity to a stack of non-dividing somatic cells called the terminal filament, and a group of somatic cells at its base called cap cells (Fig. 1A; Forbes et al., 1996a,b; Lin and Spradling, 1993). Following germline stem cell division, each daughter adopts a separate fate. One remains a stem cell at the tip of the germarium, close to the base of the terminal filament, while the other moves away from the tip and becomes a cystoblast. The cystoblast undergoes four rounds of division with incomplete cytokinesis to produce a 16-cell germline cyst. Following the

fourth cystoblast division, the progeny of the somatic stem cells invaginate from the germarium wall and encapsulate the newly formed germline cyst creating the follicle of the egg chamber. Within each germline cyst, one cell becomes the oocyte and moves to the posterior of the egg chamber while the remaining 15 cells become nurse cells (reviewed in Spradling, 1993). As the egg chamber develops, localized maternal factors are laid down in the oocyte that determine the future axes of the embryo (reviewed in St Johnston and Nüsslein-Volhard, 1992).

In many organisms, the formation of germline cells in the embryo is associated with specialized cytoplasm, known as germ plasm, which is thought to contain localized germline determinants (reviewed in Rongo and Lehmann, 1996). In *Drosophila*, the machinery that localizes the germ plasm, and is therefore required for germline formation, also localizes the posterior determinant in embryonic patterning, the Nos protein (Rongo and Lehmann, 1996). In fertilized eggs, translation of posteriorly localized *nos* RNA creates a posterior protein gradient (Gavis and Lehmann, 1994; Dahanukar and Wharton, 1996; Gavis et al., 1996; Smibert et al., 1996). Nos, together with another protein Pumilio (Pum), represses the translation of maternal *hunchback* (*hb*) mRNA in the posterior of the embryo allowing abdominal development (Barker et al., 1992; Tautz and Pfeifle, 1989). Pum contains a novel RNA-binding domain and binds specifically to *nanos* response elements (NREs) in the 3′ UTR of the *hb* mRNA (Wharton and Struhl, 1991; Murata and Wharton, 1995; Zamore et al., 1997). Since Pum protein is ubiquitously distributed in the embryo but only active in the repression of *hb* translation in the posterior, it has been suggested that interaction of RNA-bound Pum with posteriorly localized Nos mediates translational repression (Murata and Wharton, 1995).

Nos protein localized at the posterior pole of the fertilized egg is incorporated into the germ cells as they form. While Nos is absent from the rest of the embryo, it is present in the germ cells throughout embryogenesis (Wang et al., 1994; Moore et al, 1998). In the adult ovary, Nos protein is detected in the germline stem cells and developing cystoblasts (Fig. 1C; Wang et al., 1994). We were interested in addressing the function of *nos*, both during germ cell migration and subsequently during oogenesis. It has previously been shown that transplanted germ cells lacking *nos* activity fail to populate the embryonic gonad (Kobayashi et al., 1996). We show here that maternal *nos* is essential for the migration of germ cells following their exit from the posterior midgut. Adult females lacking *nos* activity produce very few eggs suggesting that *nos* is also required during oogenesis (Wang et al., 1994). We have analyzed in detail the effect of the loss of *nos* activity on oogenesis.

In the early embryo, Nos requires Pum activity for its role in embryonic patterning. To determine whether *pum* acts with *nos* in germline development, we analyzed the ovary phenotype of strong *pum* mutant alleles. We find that, while both *pum* and *nos* are required in the germline for its maintenance, they are expressed in overlapping but distinct domains (Fig. 1). While the focus of the *pum* phenotype is on the maintenance of germline stem cells, a critical feature of the *nos* phenotype is the arrest of germline cyst development. This raises the possibility that *nos* and *pum* may interact with different partners during oogenesis.

MATERIALS AND METHODS

Fly strains

Nanos

nos^{RC} is an intron-exon splice site mutation resulting in an unstable RNA and appears to be a protein null by protein blot (Curtis et al., 1997). *Df(3R)Dl-FX3* is a deficiency that extends over the *nos* gene. nos^{RC}/*Df(3R)Dl-FX3* is the strongest combination of *nos* alleles and may well represent the *nos* null phenotype.

nos^{BN} is a mutation resulting from a P-element inserted into the 5′ region of the *nos* gene. nos^{BN} is expressed in the germarium but not later during oogenesis, so that maternal *nos* RNA is not deposited into the egg. As a consequence this allele has a strong effect on abdomen formation but not on oogenesis (Wang et al., 1994).

Pumilio

The *pum* genomic region extends over more than 120 kb. So far a deletion that removes only the *pum* gene has not been identified. Two chromosomal aberrations, *In(3R)Msc* and *T(3,X)FC8* break within the *pum* genomic region and have previously been used to assess the maternal effect phenotype of *pum*. While these two mutations seem to lack activity for the function of *pum* in *hb* regulation, our studies suggest that *T(3,X) FC8* has residual activity for the function of *pum* during oogenesis. *In(3R)Msc* is an inversion with one break point in the 8th intron of *pum*. This mutant produces no detectable protein on western blots (Chagnovich, Barker and Lehmann, unpublished data). *In(3R)Msc* is therefore likely to be a null mutation for *pum* function.

A number of EMS-induced *pum* alleles have been identified amongst which are several mutations that show a strong effect on embryonic patterning and oogenesis. We have studied the following combinations of strong *pum* alleles: pum^{ET1}/*In(3R)Msc*, pum^{ET4}/*In(3R)Msc*, pum^{ET5}/*In(3R)Msc*, pum^{ET7}/*In(3R)Msc*, pum^{ET9}/*In(3R)Msc*, and pum^{ET1}/pum^{ET9}. The allelic combinations used here give the strongest abdominal phenotype and all result in the loss or considerable reduction of full-length Pum in protein blots. The pum^{ET9} and pum^{ET7} alleles were sequenced. pum^{ET9} was found to contain a deletion of base pairs 4224-4498 resulting in a frame shift and premature stop codon. pum^{ET7} contains an A to T switch at base pair 3890, which changes amino acid 949 to a stop codon (Chagnovich, Barker and Lehmann, unpublished data). The resulting truncated proteins in both cases lack the RNA-binding domain required for the binding of Pum to *hb* RNA (Zamore et al., 1997). We therefore suggest that pum^{ET7}/*In(3R)Msc* and pum^{ET9}/*In(3R)Msc* flies most likely present the *pum* lack-of-function phenotype. Both genotypes are subvital. Ovaries from pum^{ET7}/*In(3R)Msc* and pum^{ET9}/*In(3R)Msc* flies that survive show a similar phenotype to the other combinations analyzed here suggesting that the defects that we are seeing in all cases may indeed reflect the phenotype produced by complete loss of *pum* activity.

Numbers of ovaries analyzed for different genotypes and ages

Genotype	Age at which ovaries were dissected		
	0-5 days	8-16 days	21-40 days
nos^{RC}/*Df(3R)Dl-FX3*	20	15	60
pum^{ET1}/*In(3R)Msc*	20	50	30

Maternal clones

yw; FRThb^{FB}*nos* ^{BN} females were crossed to *yw hsFLP; FRTovo*^{D} males (both a gift from Claude Desplan), and their progeny heat shocked as larvae to induced FLP-mediated recombination (Chou and Perrimon, 1992). Larvae were heat shocked in a 37°C water bath for 1 hour twice a day from 2 days after egg lay until pupation. *yw hsFLP; FRThb*^{FB}nos^{BN}/*FRTovoD* females were collected and crossed to either nos^{RC}/*Df(3R)Dl-FX3* or wild-type males. Because of the presence of

a copy of ovo^D in these females, only $hb^{FB}nos^{BN}$ homozygous germline clones can give fertile ovarioles.

Germ cell transplants

Germ cell transplants were carried out as described by Ingham and Forbes (1993). To test for the requirement for *nos* in the germline, hosts were 2- to 3-hour-old embryos from a cross between $nos^{RC}/TM3$ females and *Df(3R)Dl-FX3/TM3* males. Germ cell donors were embryos of the same age from a stock marked with *yellow* (*y*) and *white* (*w*) markers. To test the germline requirement for *pum*, embryos from the cross $pum^{ET1}/TM3$ and *In(3R)Msc /TM3* were used as hosts and the donor embryos were marked with *w.*

Histochemical staining

Ovary staining

Antibody staining of ovaries was carried out as described by Lin et al. (1994). An anti-Pum polyclonal antibody directed against the central part of the protein, amino acids 408-883, was used at a dilution of 1:1000 (Barker and Lehmann, unpublished; Zamore et al., 1997), anti-Vasa polyclonal antibody (a gift from A. Williamson) was used at 1: 5,000, anti-alpha Spectrin polyclonal antibody (a gift from D. Branton) was used at 1: 400 and anti-Nos polyclonal antibody was used at 1:10,000 (Wang et al., 1994). In all cases, a Cy3-conjugated donkey anti-rabbit secondary antibody from Jackson ImmunoResearch Laboratories was used at 1:400. DAPI staining was carried out as described by Lin et al. (1994). All samples were mounted in 50:50 PBS:Glycerol containing 2.5% DABCO (Sigma).

Embryo staining

Embryos were stained as described by Eldon and Pirrotta (1991). Anti-Vasa polyclonal antibody (A. Williamson) was used at 1:10,000, followed by a biotinylated secondary antibody (Jackson ImmunoResearch Laboratories) at 1:400. Antibody detection was then done using Vectastain ABC kit to produce avidin-biotin-HRP complex.

RESULTS

Maternally provided Nanos protein is required for normal germ cell migration

High levels of maternal Nos are incorporated into the germ cells in blastoderm embryos and can be detected until stage 15 of embryogenesis (Wang et al., 1994). New synthesis of *nos* RNA in germ cells is not detectable until the first larval instar (Rongo and Lehmann, unpublished data). Since embryos from homozygous *nos* mutant females form germ cells but lack abdomen, it is difficult to study the effect of loss of maternal Nos activity on germ cell behavior. Kobayashi et al. (1996) overcame this problem by transplanting germ cells from embryos lacking maternal *nos* activity into wild-type embryos. They showed that none of the transplanted mutant germ cells reach the embryonic gonad and that the absence of Nos product results in premature transcription of certain late germ cell markers (Kobayashi et al., 1996). However, the transplantation process may disturb the development of the host embryo and affect germ cell behavior and only very few embryos can be observed by this method. To overcome these problems, we used a genetic method to study, in detail, the behavior of germ cells lacking maternal *nos*.

In wild-type embryos, *nos* is required to repress maternal *hb* RNA translation in the posterior of the embryo and thereby allow abdomen formation (Tautz and Pfeifle, 1989; Wharton and Struhl, 1991). It has been shown that embryos from females that lack both *nos* and *hb* activity in the germline give rise to normally segmented embryos as long as a wild-type copy of *hb* is provided by the sperm (Hülskamp et al., 1989; Irish et al., 1989; Struhl, 1989). Adult flies homozygous for *nos* and *hb* will not survive since *hb* homozygotes die as embryos (Lehmann and Nüsslein-Volhard, 1987). Thus, to produce normally segmented embryos that lack Nos protein in their germ cells, we studied the progeny of germline clones mutant for both *hb* and *nos*. These were made using the *FLP/FRT/ovoD* system (Chou and Perrimon, 1992). We used hb^{FB}, a null mutation (Hülskamp et al., 1994) and nos^{BN}, which removes *nos* function from the embryo but not the ovary (Materials and Methods).

To study the effect of loss of both maternal and zygotic Nos product on germ cell migration, females with $hb^{FB}nos^{BN}$ mutant germline clones were crossed to males transheterozygous for a *nos* null mutation and a deficiency that deletes the gene ($nos^{RC}/Df(3R)Dl-FX3$). Germ cells were visualized by staining with an anti-Vasa antibody.

Germ cells are formed in embryos lacking *nos* activity indicating that *nos* function is not required for their formation. However, we show that *nos* activity is essential for the successful migration of these germ cells. In embryos lacking maternal Nos, defects in germ cell migration are seen from stage 10 onwards. Following the exit of germ cells from the posterior midgut pocket, germ cells fail to migrate over the surface of the gut and instead cluster tightly together on the outer gut surface (Fig. 2B). In many embryos most of the germ cells remain in a large cluster associated with the distal tip of the posterior midgut as it extends anteriorly during embryonic development (Fig. 2D). In some embryos, a few germ cells do get into the mesoderm. These tend to form small clumps in the lateral abdominal mesoderm. Double staining with anti-Vasa antibody and a gonadal mesoderm marker shows that these cells are occasionally associated with the gonadal mesoderm (data not shown).

Not only the behavior, but also the morphology of the germ cells seems to be altered in the absence of maternal Nos activity. In contrast to wild-type germ cells, which are of consistently large size with a smooth surface and evenly stained with anti-Vasa antibody, *nos* mutant germ cells are of varying size and often have an irregular surface and uneven Vasa staining. Also the mutant germ cells seem to cluster very tightly together as soon as they exit the midgut whereas, in wild-type embryos, this tight association between germ cells is only seen following their association with the gonadal mesoderm at a later stage in embryogenesis.

To assess whether zygotic expression of *nos* in germ cells can rescue the effects of loss of maternal product, females with $hb^{FB}nos^{BN}$ mutant germlines were crossed to wild-type males. The same defects in migration are seen in all germ cells lacking maternal Nos activity independent of the paternal genotype. This indicates that zygotic *nos* expression cannot compensate for the loss of maternal Nos.

To test whether the few germ cells that reach the somatic gonad in embryos lacking maternal *nos* activity can go on to populate the adult gonads, ovaries and testes were dissected from flies that developed from embryos derived from $hb^{FB}nos^{BN}$ maternal clones. A total of 70 females were dissected 1-4 days following eclosion. Staining with anti-Vasa antibody showed that all of these ovaries completely lack

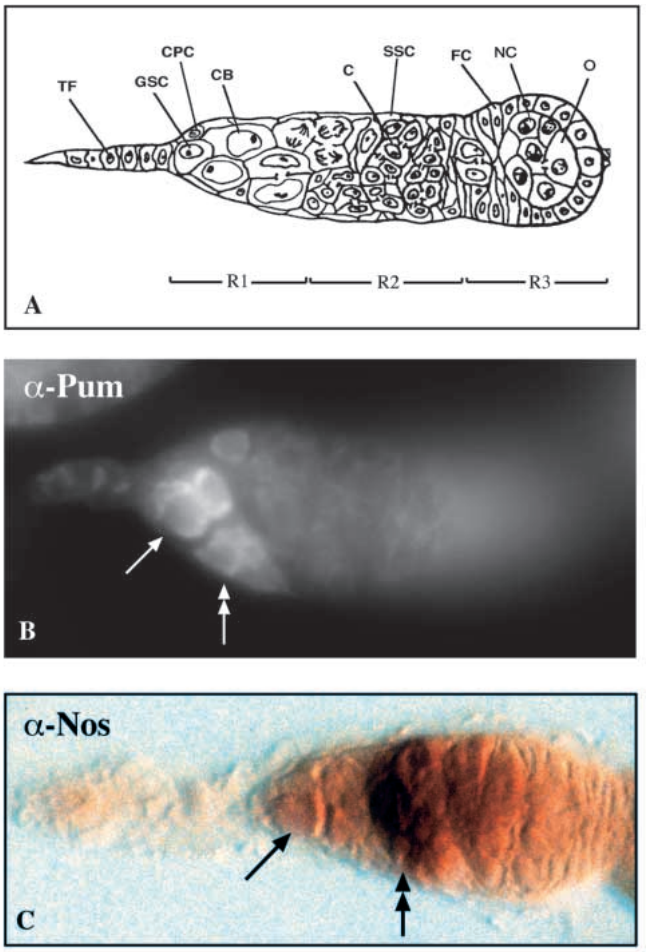

Fig. 1. Nanos and Pumilio expression in the germarium. (A) Diagram of a germarium after Forbes et al. (1996a). The germarium is divided into three regions: In region 1 (R1), 2-3 germline stem cells (GSC) are located adjacent to the somatic cap cells (CPC) at the base of the terminal filament (TF). When they divide they rejuvenate themselves and give rise to cystoblasts (CB). The rest of region 1 contains 3-5 dividing cystoblasts. In region 2 (R2), 3-5 germline cysts (C) undergo encapsulation by the progeny of somatic stem cells (SSC). Region 3 (R3) contains one stage 1 egg chamber, which contains 15 nurse cells (NC) and a posteriorly localized oocyte (O), and is surrounded by follicle cells (FC). (B) Germarium stained with anti-Pum antibody. High levels of Pum are present in the germline stem cells (single-headed arrow) and lower levels of protein are seen in dividing cystoblasts (double-headed arrow). (C) Germarium stained with anti-Nos antibody. The highest level of Nos protein is in germline cysts in region 2 (double headed arrow), levels decline as the cysts are encapsulated by somatic cells. Single headed arrow points to germline stem cells which express low levels of Nos (Wang et al., 1994).

germline irrespective of whether or not the flies carried a wild-type copy of the *nos* gene from their father (Fig. 2E). In contrast, females of the same genotypes arising from embryos with maternal Nos do contain germline and produce eggs (Fig. 2G). Similarly, testes from males arising from homzygous $hb^{FB}nos^{BN}$ germline clones also lack germline, while testes from homozygous *nos* males derived from heterozygous mothers are populated with germ cells (data not shown). This indicates that, in embryos lacking maternal Nos, the few germ cells that appear to associate with the somatic embryonic gonad are unable to incorporate into the adult gonad even in the presence of a zygotically active copy of the gene.

Zygotic *nanos* function is required for the proliferation and viability of germ cells in the germarium

In adult females, Nos protein is detected at high levels in germline cysts in region 2 of the germarium (Fig. 1C). Lower levels of Nos protein are also observed in the germline stem cells and dividing cystoblasts (Wang et al., 1994). It has been shown that females transheterozygous for strong *nos* alleles lay a small number of eggs early in life but soon become completely sterile suggesting an important function for *nos* activity during oogenesis. To further investigate the basis of this sterility and the role of *nos* during oogenesis, ovaries from $nos^{RC}/Df(3R)Dl\text{-}FX3$ (Materials and Methods) females ranging from 1 day to 40 days old were dissected and stained with anti-Vasa antibody to reveal the germline cells.

We have found that the sterility of *nos* mutants results from a loss of germline cells from the ovary. This seems to occur in three ways. Firstly, some ovarioles in mutants appear to never contain germline. In newly emerged $nos^{RC}/Df(3R)Dl\text{-}FX3$ females germ cells are absent from about 50% of ovarioles (Fig. 3A,A2). Thus, zygotic *nos* activity appears to be required for the efficient inclusion of germ cells in the developing adult ovary. Secondly, in a few of the ovarioles that do initially contain germline, germline stem cells are not established and the germ cells that are present develop as cystoblasts. This results in ovarioles containing one or two egg chambers attached to an empty germarium (Fig. 3B,B2, C,C2). However, early differentiation of the germline as cystoblasts is not the typical phenotype of *nos* mutants, rather, germline stem cells appear to be established but then disappear 1-3 weeks after eclosion. In newly eclosed *nos* mutant females most of the germaria that contain germline have putative germline stem cells (Fig. 3A,A2, B,B2). However, these germaria fail to give rise to the normal succession of developing egg chambers. This is most strikingly seen in *nos* females aged for 10 days following eclosion. In these flies, many ovarioles contain a single mature egg associated directly with a germarium that contains germline cells in the tip (Fig. 3D,D2). This indicates that, in *nos* mutants, germline cysts rarely develop into egg chambers after the first few chambers have left the germarium.

In *nos* mutant germaria putative germline stem cells are seen adjacent to the cap cells at the base of the terminal filament and dividing cystoblasts are present in region 1 (Figs 3B, 6B). However, regions 2 and 3 contain fewer germline cysts than wild type and region 3 is frequently void of germline (Figs 3A,B, 6B). This phenotype suggests that the focus of *nos* function in oogenesis may lie in the development of germline cysts rather than the maintenance of germline stem cell identity. The reduction in the number of egg chambers produced by *nos* mutant ovarioles can partly be accounted for by the death of germline cysts following cystoblast division. Dying germline cysts and remnants of such cysts are seen in region 2 in some mutant germaria (Fig. 3E,E2). In addition, in many *nos* mutant germaria, region 1 contains only one or two cystoblasts and dividing cysts. This suggests that earlier stages of germline cyst development may also be retarded in *nos* mutants and that a reduction in the rate of germline stem cell proliferation may contribute to the deficit of developing germline cysts in *nos* mutants.

Putative germline stem cells are present in the germaria of many *nos* mutant ovarioles for at least 10 days following eclosion; however, ovaries from *nos* females aged for 21 days or more completely lack Vasa-staining cells (Fig. 3F). Since putative germline stem cells are present whenever germ cells are seen in germaria, and since very few ovarioles contain cysts in region 2 but lack germline cells at the tip of the germarium, we conclude that stem cells may die prematurely in *nos* mutant ovaries rather than differentiating as cystoblasts.

Our studies suggest that zygotic *nos* activity is required for the efficient incorporation of germline cells into developing ovarioles. In addition, *nos* is required in the adult ovary for the normal proliferation and viability of germline stem cells, and for the development of germline cysts.

nanos function is required in the germline

To test whether *nos* activity is required exclusively in the germline, we transplanted germ cells carrying the *yellow* and *white* markers (*yw*) into the progeny of a cross between two strong *nos* mutations, nos^{RC}/*TM3* and *Df(3R)Dl-FX3/TM3*. All emerging females were crossed to *yw* males and their offspring analyzed for the presence of *yw* progeny. *yw* progeny could only result from the incorporation of a transplanted germ cell into the host germline.

20% (n=8/32) of the nos^{RC}/*Df(3R)Dl-FX3* females were fertile and produced only offspring derived from the transplanted *yw* germ cells, indicating that wild-type germ cells can rescue the *nos* ovary phenotype. The ovaries of five of these fertile mutant females were dissected and DAPI stained 2-3 weeks after eclosion. All contained at least one ovary that looked wild type (Fig. 3G). In contrast, 46 ovaries were dissected from 3-week-old mutant females from the cross that produced the hosts but that had not received germ cells. In these ovaries, no normal ovarioles were found, not even a single normal developing egg chamber (Fig. 3F). Rescue of the *nos* ovary phenotype by wild-type germ cells demonstrates that *nos* function is required in the germline for oogenesis.

In each of the rescued ovaries from fertile *nos* mutant females, the transplanted *yw* germ cells fill a larger proportion of the ovarioles than do *yw* germ cells in the ovaries of their heterozygous siblings. In most of the rescued mutant ovaries, transplanted germ cells populate the entire ovary. In 5/7 rescued mutant ovaries, all 15-16 ovarioles contained germline cells and a normal number of developing egg chambers. In two rescued ovaries, a small proportion of the ovarioles (2/15 in one ovary and 5/15 in the other) lacked germline. In contrast, the contribution of transplanted *yw* germ cells to the germline in heterozygous hosts was less extensive. Only 10-20% of the progeny from each heterozygous host containing transplanted *yw* germ cells arose from the transplanted germ cells. This suggests that transplanted wild-type germ cells populate the ovary more efficiently in embryos whose endogenous germ cells lack *nos* activity than in embryos with germ cells containing a zygotically active copy of the *nos* gene. This supports the idea that germ cells lacking zygotic *nos* activity are compromised in their ability to populate the ovary.

Pumilio is required for germline stem cell maintenance

pum is required for *nos* function in pattern formation. Given the strong oogenesis phenotype in *nos* mutant females, we were interested to determine whether *pum* mutants have a similar effect to *nos* in the ovary. Recently, Lin and Spradling (1997) showed that a group of P-element insertion mutants called $pum^{ovarette}$ (pum^{ovt}) result in the rapid loss of germline stem cells from the adult ovary. While these mutants fail to complement the abdominal phenotype of some previously identified *pum* mutants, the analysis of the oogenesis phenotype leaves it unclear as to whether pum^{ovt} and *pum* are

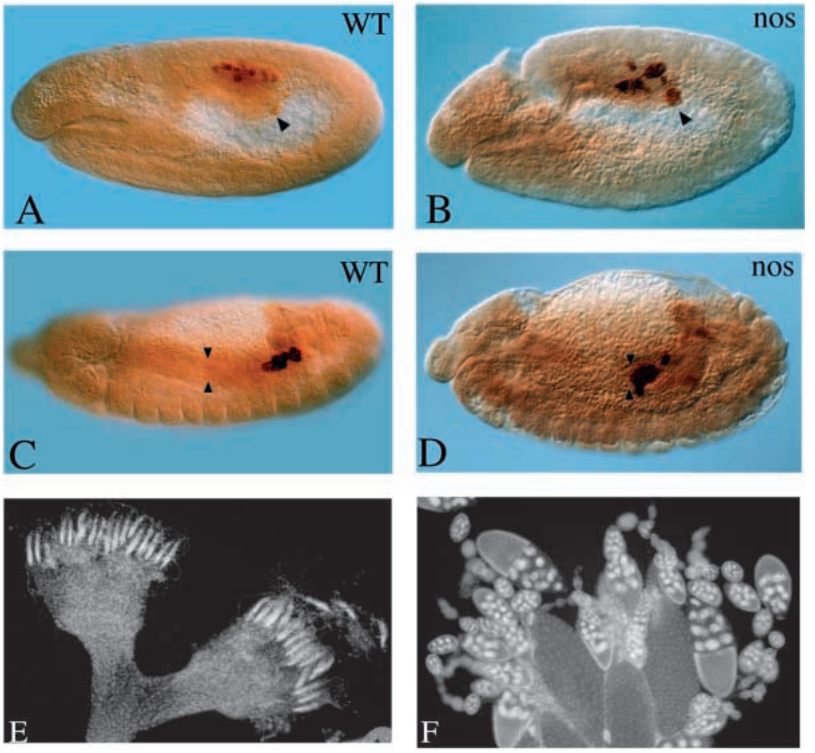

Fig. 2. Nanos is required for germ cell migration and population of the gonad by germ cells. (A-D) Embryos stained with anti-Vasa antibody marking germ cells in brown. Arrowheads point to the distal end of the posterior midgut in all panels. (A) Wild-type stage 11 embryo with germ cells moving off of the dorsal side of the gut into the mesoderm. (B) Stage 11 embryo from a *hb nos* homozygous maternal clone crossed to a nos^{RC}/*Df(3R)FX3* male. Germ cells are clustered tightly together in clumps on the basal surface of the gut. (C) Stage 13 wild-type embryo, germ cells are associated with the gonadal mesoderm in abdominal segment 5 and 6. (D) Stage 13 embryo from a *hb nos* maternal clone. Most germ cells are tightly clustered and remain stuck to the distal end of the posterior midgut (arrowheads). (E,F) DAPI-stained ovaries from 2- to 4-day-old nos^{RC}/nos^{BN} flies derived from mothers with *hb nos* maternal clones (E) or heterozygous mothers (F). In flies that lacked maternal Nos as embryos, ovaries completely lack germ cells. (E) shows such an ovary. This ovary was also stained with anti-Vasa antibody, but no Vasa-positive germ cells were present. In contrast, in nos^{BN}/nos^{RC} flies from embryos with maternal Nos activity ovaries are fully populated with germ cells (F).

the same gene (Lin and Spradling, 1997, see Discussion). To clarify the role of *pum* in oogenesis, we have analyzed the ovary phenotype of a number of different combinations of the strongest *pum* alleles. While the phenotypes of the different combinations are quite similar, we will concentrate in the following description of the *pum* ovary phenotype on the $pum^{ET1}/In(3R)Msc$ allele combination (Materials and Methods).

Ovaries from $pum^{ET1}/In(3R)Msc$ females were dissected from 1 day to 35 days after eclosion and stained with an anti-Vasa antibody to reveal the germline (see Materials and Methods for numbers). While mutant females lay some eggs during their first week of life, by 10 days after eclosion, their ovaries are completely depleted of germline (Fig. 5A). This loss of germline over time seems to be a shared phenotype between *nos* and *pum* mutant ovaries. However, germline stem cells are lost in *pum* mutants earlier and more consistently than in *nos* mutants suggesting that the mechanisms by which the two genes affect germline stem cells may be different.

In newly eclosed $pum^{ET1}/In(3R)Msc$ females, ovaries occasionally completely lack germline (Fig. 4B,B2); however, in most ovaries, the majority of the ovarioles contain Vasa-staining germline cells. Among 15 ovaries analyzed, 30% of the ovarioles lack germline. This indicates that, as in *nos* mutants, germ cells lacking *pum* activity may be compromised in their ability to populate the adult ovary. In $pum^{ET1}/In(3R)Msc$ ovarioles that do contain germline, germline stem cells do not appear to be maintained and instead

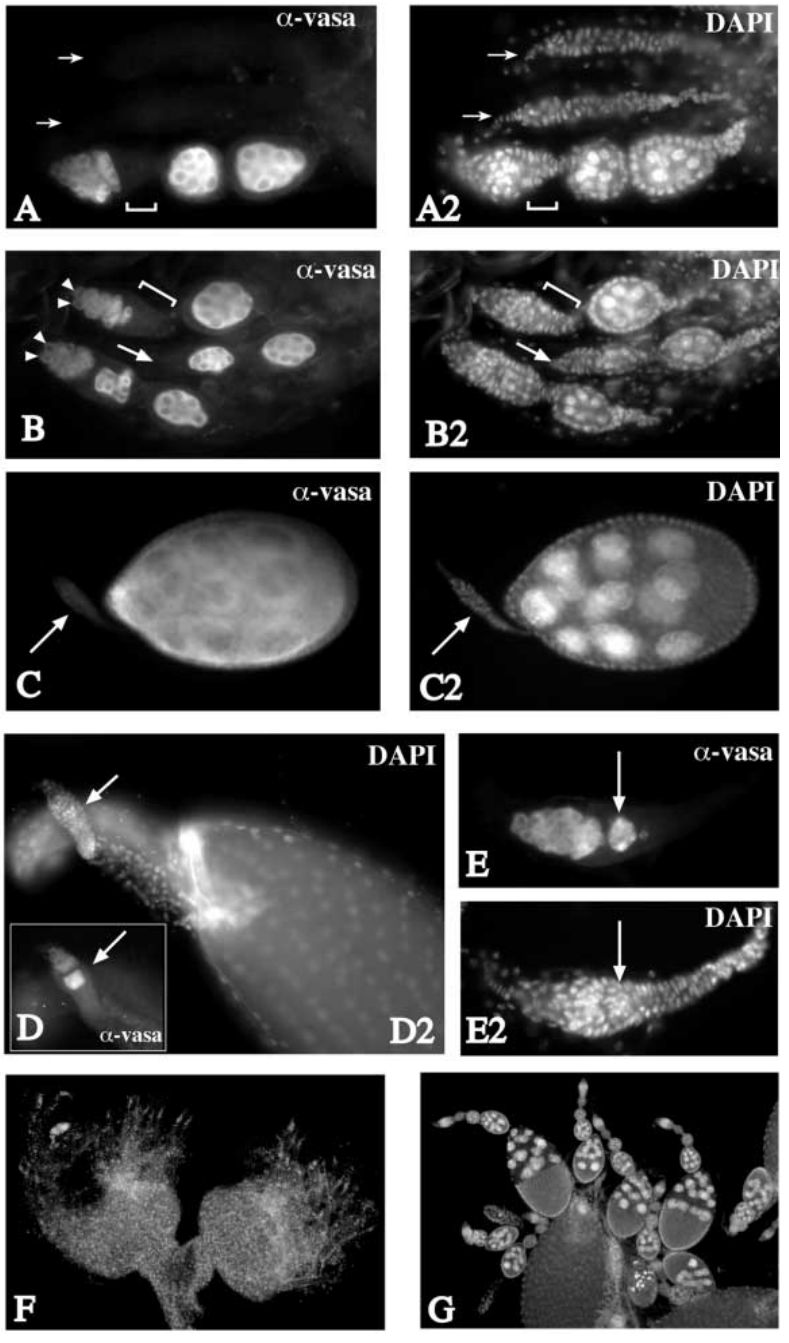

Fig. 3. *nanos* is required in the germline for viability and proliferation of germline stem cells in the adult ovary. Ovaries from $nos^{RC}/Df(3R)FX3$ females stained with anti-Vasa antibody to reveal the germline (A-E) and DAPI to show the nuclei (A2-E2). Ovarioles shown in A and B were dissected from newly eclosed *nos* mutant females. (A,A2) The upper two ovarioles (arrows) lack germline cells. The lower of the three ovarioles contains two developing egg chambers and regions 1 and 2 of the germarium are populated with germline cells. However, regions 2-3 of the germarium (bracket) lack germline cysts. (B) Arrowheads point to putative germline stem cells in the upper and the lower ovariole. However, germline stem cells are not present in the middle ovariole (arrow). (B,B2) Arrow points to the germarium tip of this ovariole. Germline cells are absent from the tip and the only germline cell remaining in this germarium is developing as an egg chamber. (C,C2) Ovariole from 5-day-old *nos* mutant female. A germarium depleted of germline cells (arrow) is attached to a developing stage 8 egg chamber. (D2,inset D) Ovariole from a 14-day-old *nos* mutant female. Germarium containing germline cells (arrow) is attached directly to a stage 14 egg chamber. (E,E2) Germarium from a newly eclosed *nos* mutant female. The most posterior of the germline cysts (arrow) appears to be dying as indicated by the cluster of pycnotic nuclei staining brightly with DAPI (E2). The small fragment of Vasa staining posterior to this dying cyst may be the remnant of an earlier cyst death. (F) Ovaries from a 23-day-old $nos^{RC}/Df(3R)FX3$ female stained with DAPI are completely depleted of germline. (G) Ovary from 17-day-old $nos^{RC}/Df(3R)FX3$ female host containing transplanted *yw* marked germ cells. These germ cells have fully populated the ovary and completely rescued the *nos* mutant phenotype.

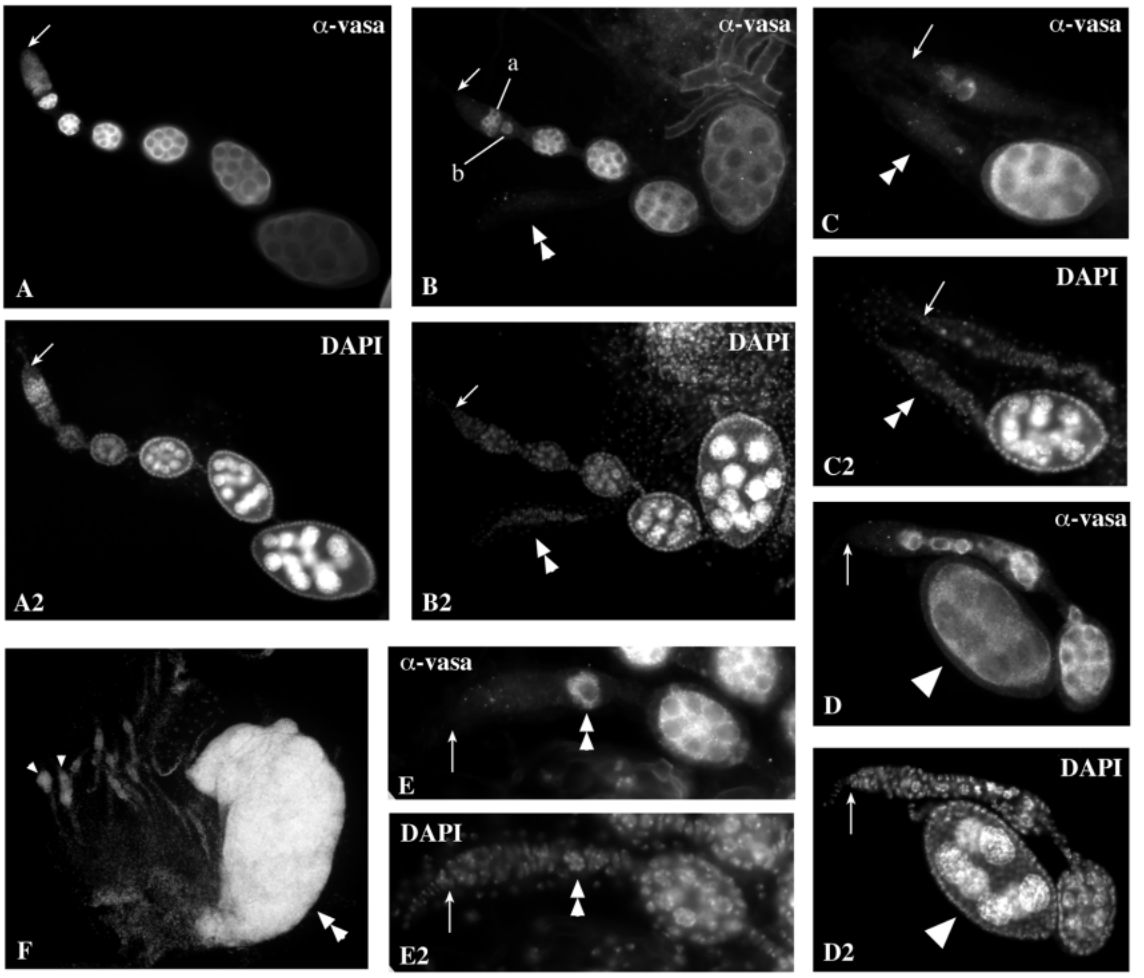

Fig. 4. *pumilio* is required in the adult ovary for the maintenance of germline stem cells. (A,A2) Wild-type ovariole stained with anti-Vasa antibody (A) and DAPI (A2). A germarium populated with germline cells is attached to a chain of progressively developing egg chambers. (B-E) Ovarioles from pum^{ET1}/ *In (3R)Msc* mutant females dissected 1-5 days following eclosion. In each panel, the single-headed arrow points to the position of the cap cells at the base of the terminal filament. In wild-type germaria (A), germline stem cells are closely associated with the cap cells. In each of the *pum* mutant ovarioles, Vasa staining germ cells are never seen adjacent to cap cells (B-E). In the upper ovariole in B, the most anterior germline cells constitute a cyst in region 2 (a), no germline cells remain in the germarium tip. Just posterior of the cyst is a single germline cell apparently developing as a nurse cell (b). Also in B and B2 is an ovariole containing no germline (double arrowhead). In *pum* mutant ovarioles egg chambers are frequently seen attached to empty germaria (C, C2 double arrowhead). This could result from the differentiation of all germline cells as cystoblasts. In some cases, the final germline cell to leave the germarium fails to divide but nevertheless develops as a nurse cell (B(b), upper ovariole C, C2 and E, E2 double arrowhead). Sometimes these final germ cells form incoherent clusters with fewer than the normal number of germline cells (D,D2). The egg chamber in D, D2 (arrowhead) contains 7 nurse cells and an oocyte. (F) pum^{ET1}/*In (3R)Msc* mutant ovary from a 35-day-old female stained with DAPI. This ovary is depleted of germline and some of the germaria are swollen (small arrowheads), while one germarium has formed an extensive tumor (double arrowhead).

the germline cells present differentiate as cystoblasts and subsequently into egg chambers. Germ cells are never seen at the very tip of the germarium in close proximity to the terminal filament and cap cells (Fig. 4B-E, 6C). In most germaria, regions 1 and 2 are empty (Fig. 4B, 6C). Typically ovarioles from newly eclosed mutant females contain only 1-3 clusters of germline cells in the germarium and 1-3 developing egg chambers.

In most *pum* mutant ovarioles, all of the germline cells that we detect by Vasa staining behave as cystoblasts and give rise to normal egg chambers. As a result, empty germaria are observed attached to normally developing egg chambers (Fig. 4C,C2). However, in some ovarioles, while the first few germline cysts differentiate normally, germ cells that leave the germarium later behave aberrantly. These germ cells appear to take on cystoblast fate but fail to complete the normal four rounds of division resulting in abnormal germline cysts consisting of a few or even individual germ cells (Fig. 4D,E). In some cases, germline cysts lose their compact shape and become elongated and irregularly shaped (Fig. 4D). This is reminiscent of germline cysts in ovaries in which somatic cell proliferation is increased (Forbes et al., 1996a).

18 days after eclosion some of the now germline-less germaria appear swollen (Fig. 4F, 5A). By 35 days after eclosion, some ovarioles have become massively tumorous. Such tumors range in size from a slightly swollen germarium

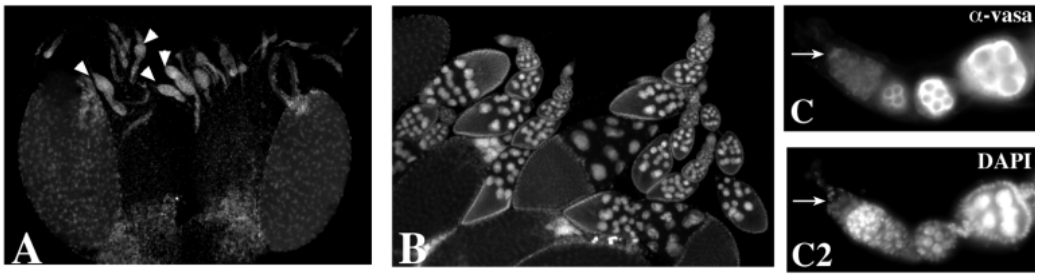

Fig. 5. The *pumilio* ovary phenotype is rescued by the expression of *pumilio* cDNA, and by transplanted wild-type germ cells. A and B are DAPI stained to show nuclei. (A) Ovary from a 13-day-old $pum^{ET1}/In(3R)Msc$ female, with two withheld stage 14 egg chambers, otherwise depleted of germline. Some of the germaria are swollen (arrowheads). (B) Ovary from a 13-day-old $pum^{ET1}/In(3R)Msc$ female carrying a transgene containing *pum* cDNA under the regulation of the *nos* promoter, this ovary appears completely wild-type. (C,C2) The tip of an ovariole from a 13-day-old $pum^{ET1}/In(3R)Msc$ female containing transplanted wild-type germline. Arrow points to the position of the cap cells at the tip of the germarium. Large germline cells, presumably germline stem cells, are located next to these cap cells. These germline stem cells appear to give rise to a normal succession of developing germline cysts and egg chambers.

to a mass of densely packed small cells that is the size of the whole ovary (Fig. 4F). These tumors do not stain with anti-Vasa antibody suggesting that they are somatic rather than germline in origin. It therefore appears that, in the absence of *pum* activity, somatic cells overproliferate and form tumors. Such somatic overproliferation appears to be a specific effect of loss of *pum* activity rather than a secondary effect of loss of germline as similar tumors are not seen in *nos* mutant ovarioles that also lose their germline (Fig. 3F). In addition, Margolis and Spradling (1995) have shown that, in $oskar^{301}$ mutant females, which lack germline throughout development, somatic stem cell division continues, but somatic overproliferation resulting in tumors is not reported.

Our analysis suggests that *pum* is required for the maintenance of the germline stem cells. This is consistent with the distribution of Pum protein in wild-type ovaries. Pum protein is highly enriched in the stem cell population at the tip of each germarium while we detect low levels of Pum in the dividing cysts (Fig. 1B). Pum protein was also detected at low levels throughout the somatic cells of the ovary although the specificity of this staining remains unclear (data not shown).

pumilio function is required in the germline

We have found that a number of different combinations of strong *pum* alleles result in the failure to maintain germline stem cells in the adult ovary with the consequent loss of the germline from the ovary. While this phenotype is very similar to that recently described by Lin and Spradling (1997) for pum^{ovt} alleles, particular allelic combinations tested in their study show a different phenotype than observed under our genetic conditions (see Discussion). Since the exact nature of the pum^{ovt} alleles and their effect on *pum* function is not known, we wanted to confirm that, in our mutant allele combinations, it is the lack of *pum* activity that causes the ovary phenotype that we observe. We therefore used a *pum*-mini-gene that expresses the *pum* cDNA under the regulation of *nos* 5′ sequences and which was previously shown to complement the maternal effect phenotype of *pum*. (Barker et al., 1992).

Ovaries from 12-day-old $pum^{ET1}/In(3R)Msc$ females lacking the transgene were dissected and found to be totally depleted of germline (Fig. 5A). In contrast, females carrying the transgene had productive ovaries that were wild type in appearance (Fig. 5B). Since the ovary phenotype observed in $pum^{ET1}/In(3R)Msc$ females is complemented by the expression of the *pum* cDNA construct, we conclude that the mutant phenotype described is due to loss of *pum* activity. Furthermore, this result also suggests that the requirement for *pum* for germline stem cell maintenance is restricted to the germline since transcription of the *pum* cDNA is directed by the *nos* promoter, which drives germline-specific expression.

To further test whether *pum* activity is indeed germline autonomous, we transplanted germ cells marked with the *white* (*w*) marker into the progeny of the cross $pum^{ET1}/TM3 \times In(3R)Msc/TM3$. Both homozygous and heterozygous females that emerged were crossed to *w* males and their offspring analyzed for *w* flies, which can only be generated by transplanted germ cells. 25% (n=8/32) of $pum^{ET1}/In(3R)Msc$ mutants were fertile and produced all *w* offspring, and 19% (n=8/43) of heterozygous females gave rise to some *w* offspring. Only two of the fertile $pum^{ET1}/In(3R)Msc$ mutant females lived for longer than a week following eclosion and both remained fertile during this time. One of these females was dissected 14 days after eclosion. This female had one ovary that completely lacked germline while the other ovary was filled with many withheld stage 14 egg chambers and contained at least 7 ovarioles in which the germaria appeared normal. Putative germline stem cells were observed adjacent to the basal cells of the terminal filament in these germaria, and dividing cystoblasts and germline cysts were seen in regions 1 and 2 (Fig. 5C,C2). These germaria were associated with a chain of three to four developing egg chambers. The presence of putative germline stem cells in this *pum* mutant ovary containing *w* germ cells strongly suggests that *pum* activity in the germline is sufficient for the maintenance of germline stem cells. However, this does not rule out an additional role for *pum* in the soma during oogenesis.

nos and *pum* have different effects on germline stem cells

pum and *nos* mutant flies both show a dramatic reduction in the number of eggs produced. The function of both genes seems to be required for germline cells to populate the adult ovary and both genes act in the germline. However, in those cases where germline cells populate mutant ovarioles, the germline phenotypes of *nos* and *pum* differ. The failure of germ cells to take on stem cell fate in *pum* mutants results in germaria from newly eclosed flies in which region 1 lacks

germ cells (Fig. 6C,C2). In contrast, in *nos* mutants, putative germline stem cells and dividing cystoblasts are present in region 1 for a week or more following eclosion, but regions 2 and 3 tend to be void of germline cysts suggesting arrest of cyst development following cystoblast division (Fig. 6B,B2).

A characteristic feature of germline stem cells is the presence of a spectrosome, a large organelle containing several cytoskeletal components including Spectrin. The spectrosome is located at the stem cell membrane where it associates with the basal cell of the terminal filament or cap cell (Fig. 6D,D2) (Lin and Spradling, 1993). In addition, the fusome, which connects the progeny of dividing germline cysts, has been shown to accumulate Spectrin (Lin et al., 1994). We analyzed Spectrin distribution in *nos* and *pum* mutant ovaries to further characterize the *nos* and *pum* oogenesis phenotypes.

Consistent with the differences in the effect on early oogenesis, we observe differences in the distribution of Spectrin when comparing *pum* and *nos* mutant germaria. In *nos* mutants, very small spectrosomes are seen in the germline stem cells closely associated with the cell membrane adjacent to the cap cells (Fig. 6E,E2). Furthermore, the amount of Spectrin associated with the fusome in the dividing germline cysts is greatly reduced in *nos* mutants (Fig. 6E,E2). This phenotype suggests that, while stem cells are established in *nos* mutants, they are not entirely normal. In *pum* mutant germaria, Spectrin-staining dots, which are almost as large as in wild type but more irregularly shaped, are seen in the most anterior germline cells. However, in contrast to wild-type stem cells, these spectrosome-containing cells are not associated with the basal cells of terminal filaments or cap cells (Fig. 6F,F2; Lin and Spradling, 1997). This is consistent with the failure to maintain germline stem cells at the germarium tip in *pum* mutants.

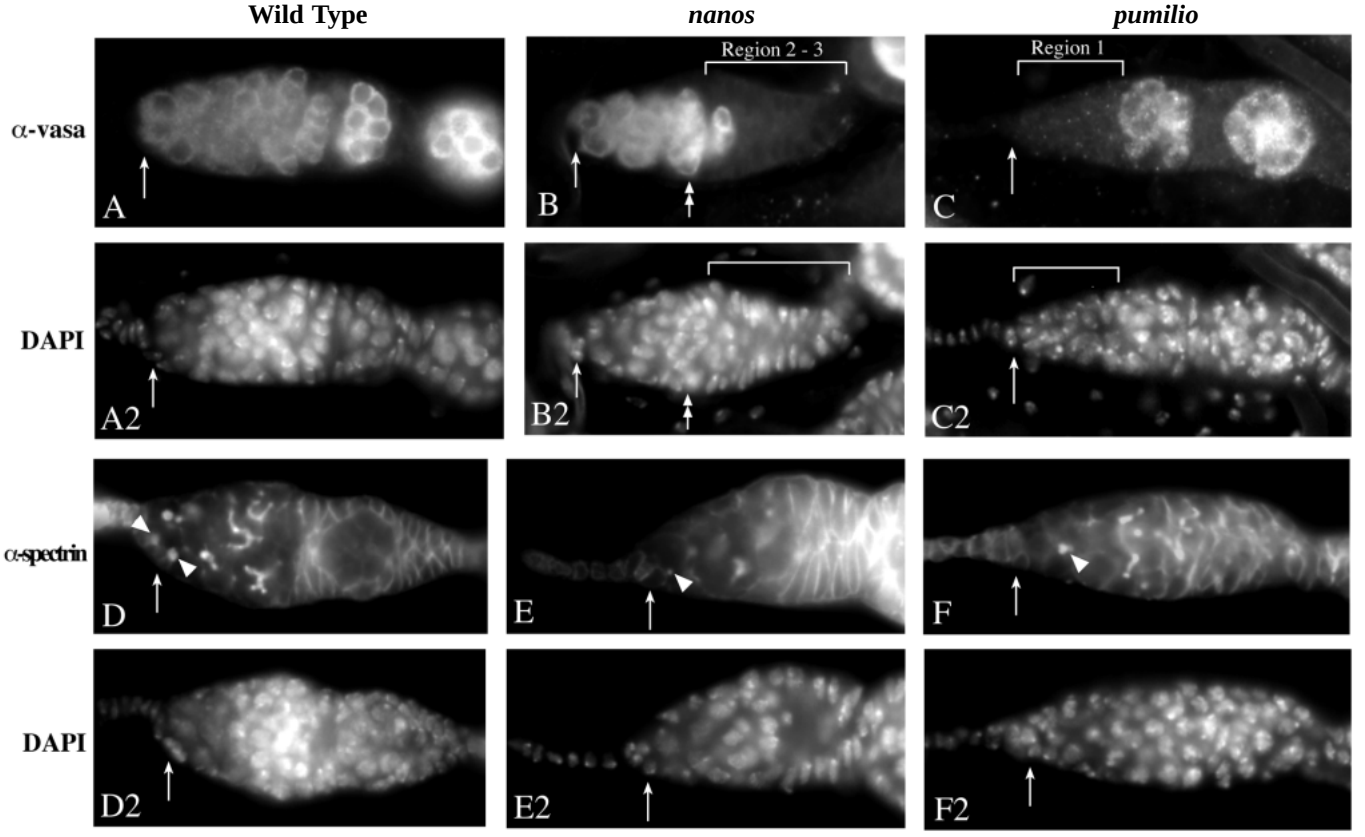

Fig. 6. *nanos* and *pumilio* mutant germaria have different phenotypes. Germaria from 1- to 3-day-old wild-type (A,A2,D,D2), $nos^{RC}/Df(3R)FX3$ (B,B2,E, E2), and $pum^{ET1}/In(3R)Msc$ (C,C2,F,F2) females are shown. In each picture the small arrow points to the position of the cap cells. (A) Wild-type germarium, stained with anti-Vasa. Three putative germline stem cells are in close proximity to the basal cell of the terminal filament and the adjacent group of cap cells. (B) Anti-Vasa-stained germarium from 3-day-old $nos^{RC}/Df(3R)FX3$ female. Large putative germline stem cells are seen adjacent to the cap cells. Region 1 contains fewer dividing cystoblasts and cysts than wild type, region 2-3 lacks germline. Cyst development appears to arrest in RZ (double arrow). (C) Anti-Vasa-stained germarium from a 2-day-old $pum^{ET1}/In(3R)Msc$ female. No germline cells are present at the tip of the germarium, and the last two germline cells have taken on cystoblast fate and are developing as germline cysts. (D) Wild-type germarium stained with anti-Spectrin, the nuclei of the cap cells are clearly seen in D2 (arrow) and their membranes are outlined by Spectrin in (D, arrow). Large aggregates of Spectrin are seen associated with these cap cells (arrowheads). (E) Anti-Spectrin-stained germarium from 3-day-old $nos^{RC}/Df(3R)FX3$ female. Nuclei of the cap cells can be seen in E2 (arrow) and their membranes stained with Spectrin (E, arrow). A tiny spectrosome is seen in close proximity to one of these cap cells (E, arrowhead). (F) Anti-Spectrin-stained germarium from a 2-day-old $pum^{ET1}/I(3R)Msc$ female. An aggregate of Spectrin (arrowhead) is seen at a distance from the cap cells consistent with the movement of germ cells away from the germarium tip.

DISCUSSION

Nanos is required for germ cell migration

Nos activity is required for the migration but not formation of germ cells. Maternal Nos protein is sequestered to the germ cells as they form. Germ cells lacking maternal Nos exit the posterior midgut, but then cluster together on the outer surface of the gut and fail to migrate into the mesoderm.

A number of mutants affecting germ cell migration have been isolated (Moore et al., 1998; Zhang et al., 1996) but, unlike *nos*, all seem to be required in the soma and none result in an immediate cessation of migration as soon as the germ cells exit the gut. Many of these mutants appear to be involved in endoderm and mesoderm specification, implying that it is essential for germ cells to recognize particular somatic cues in order to migrate normally. The failure of germ cells lacking maternal *nos* activity to migrate could result from a failure to express the appropriate receptors for these somatic guidance cues, or alternatively a failure to initiate a migratory state. Both of these could be a consequence of the premature differentiation of *nos* mutant germ cells. Kobayashi et al. (1996) have shown that, in the absence of maternal *nos* activity, certain P-element enhancer traps normally expressed in the germ cells following gonad coalescence are transcribed prematurely. This suggests that, in some respects, *nos* mutant germ cells behave as if they are already associated with the somatic gonad while they are still on the surface of the gut.

In abdomen specification, *nos* acts in combination with *pum* to repress the translation of maternally deposited *hb* RNA. Pum protein is distributed throughout the early embryo and is incorporated into the germ cells when they form (Macdonald, 1992). It is therefore possible that Nos acts with Pum in the germ cells to repress translation of maternally deposited transcripts.

The only putative target known for Nos in the germ cells is maternal *cyclin B* transcript. High levels of maternal *cyclin B* mRNA are incorporated into germ cells as they form and can be detected in the germ cells until stage 13 (Whitfield et al., 1989). However, only very low levels of Cyclin B protein are present in germ cells until stage 15, the time when expression and translation of zygotic transcripts can be detected (Whitfield et al., 1989). The absence of Cyclin B protein from the germ cells may be a consequence of translational repression of the maternal message. This *cyclin B* message has translational control elements (TCEs) similar to the NREs in *hb* and *bcd*, which mediate translational repression via the binding of *pum* and interaction with *nos* (Dalby and Glover, 1993). It is therefore possible that the TCEs fulfill a similar role in the repression of translation of maternal *cyclin B* message and that the abnormal behavior of germ cells in embryos from nos^{RC}/*Df(3R)Dl-FX3* maternal clones reflects premature translation of *cyclin B*. Prematurely high levels of Cyclin B protein may cause germ cells to arrest at an abnormal point in the cell cycle, possibly contributing to their failure to migrate and premature differentiation.

Although zygotic expression of *nos* cannot rescue the effect of loss of maternal *nos* activity on germ cell migration, the presence of maternal *nos* activity alone is not sufficient for the efficient population of the adult ovary by germ cells. Loss of zygotic *nos* activity appears to reduce the efficiency with which germ cells are incorporated into germaria. In *nos* mutants only 50% of ovarioles in newly eclosed females contain any germline cells. In addition, when wild-type germ cells are transplanted into *nos* mutants, they are incorporated into germaria in preference to the endogenous *nos* mutant germ cells. This suggests that germ cells lacking zygotic *nos* activity are compromised in their ability to populate the adult ovary. *nos* activity may be required for the proliferation of germ cells to produce sufficient numbers to fully populate the adult ovary. Alternatively, *nos* may be required in order for germ cells to differentiate appropriately and interact correctly with the somatic cells of the ovary as it undergoes morphogenesis.

pumilio and *ovarette*

We have shown that a number of combinations of strong *pum* alleles give an ovary phenotype in which germline stem cells fail to be maintained and the remaining germ cells in the germarium take on cystoblast fate. We consider both *In(3R)Msc* and pum^{ET9} to be null for *pum* function, and the *In(3R)Msc/* pum^{ET9} phenotype to represent the effect of a complete loss of *pum* activity during oogenesis (Materials and Methods). This phenotype is very similar to that of the other combinations of strong *pum* alleles that we analyzed, and we have shown that this phenotype is complemented by the expression of a *pum* mini-gene, as well as by transplanted wild-type germ cells. We therefore conclude that *pum* is required in the germline for the maintenance of germline stem cell identity.

Germline stem cell fate is closely correlated with the association of a germline cell with a terminal filament or cap cell. These non-dividing somatic cells are known to specifically express many genes including the signaling molecules *wg* and *hh* (Forbes et al., 1996a,b; Lin and Spradling, 1993). It seems most likely that signals from these cells prevent adjacent germline cells from taking on cystoblast fate and thereby retain stem cell identity. *pum* may act by repressing cystoblast differentiation in response to such a signal. Alternatively *pum* may be required for germ cell-cap cell association, the failure of which results in cystoblast differentiation.

Lin and Spradling (1997) have described the phenotype of a number of P-element insertion mutations which they have shown affect the maintenance of germline stem cells. Since these fail to complement the *pum* alleles pum^{ET1}, pum^{ET3}, *In (3R)Msc* and *T(3,X)FC8* for abdomen formation they have been called $pum^{ovarette}$ (pum^{ovt}). The ovary phenotype described for pum^{ovt} is similar to that of *pum*, in that mutant ovaries are incompletely populated with germ cells, germline stem cells are not maintained and spectrosomes are reduced in size. However, there are differences. pum^{ovt} has a stronger effect on ovary morphogenesis than the *pum* alleles we have analyzed. It was reported that ovarioles are sometimes completely absent in pum^{ovt} mutant ovaries, and that germ cells frequently continue to divide without differentiation leading to large clusters of small diploid cells that subsequently die. We did not observed these phenotypes in *pum* mutant ovaries. In addition, the somatic tumors seen with our *pum* allele combinations were not reported for any of the pum^{ovt} alleles. Furthermore, most of the pum^{ovt} alleles, including the strongest allele, 2003, do not exhibit an ovary phenotype when transheterozygous to the *pum* alleles, pum^{ET1} and *In (3R)Msc*. If the ovary defects observed in pum^{ovt} were attributable solely to a loss of *pum* activity, we would expect to see the same ovary

phenotype when strong pum^{ovt} alleles are homozygous or transheterozygous to *pum* null alleles.

A possible explanation for these inconsistencies is that our combinations of *pum* alleles do not completely remove *pum* activity and that the phenotype that we observe is a weaker or partial version of that observed for strong pum^{ovt} alleles. However, by genetic and molecular criteria *In(3R)Msc* is a null mutation in *pum*, and pum^{ET1} behaves as a strong lack-of-function allele. We therefore favor alternative explanations for the complementation data. One possibility is that the pum^{ovt} alleles may not only be affecting *pum* but also another gene that has a role in early oogenesis. Since the pum^{ovt} alleles were all induced by P-element insertions, it is possible that these P-elements could affect the activity of more than one gene. In support of this explanation, Lin and Spradling (1997) report that expression of most of the pum^{ovt} P-element inserts is limited to the terminal filament, leading to the suggestion that pum^{ovt} may act in these somatic cells to maintain the stem cell identity of the underlying germ cells (Lin and Spradling, 1997). A function for *pum* in the soma cannot be ruled out, indeed the somatic tumors that we observe in *pum* mutant ovaries may be a manifestation of such a somatic requirement. However, our transplantation and complementation data suggest that the major part of the *pum* phenotype, the loss of germline stem cells, is germline dependent.

An alternative explanation for Lin and Spradling's pum^{ovt}/*pum* complementation results is that pum^{ovt} alleles are antimorphic mutations. Such mutants give a stronger phenotype when homozygous than when transheterozygous to a deletion (Muller, 1932). Many pum^{ovt} alleles including the strongest allele, 2003, have no oogenic defect in combination with *In(3R)Msc* but a very strong effect on oogenesis when homozygous. Since the RNA-binding domain of Pum is located in the C terminus of the protein, one could speculate that separation of this region from a hypothetical regulatory region could create a dominant negative acting protein. A dominant negative form of Pum might explain both the severity of the pum^{ovt} phenotype and also its divergence from the *pum* phenotype.

nanos and *pumilio* may have overlapping and distinct functions during oogenesis

In the adult ovary, *nos* and *pum* are both required in the germline for the maintenance of germline stem cells. In both mutants, germline stem cells are lost from the germarium over time; however, this occurs later in *nos* than in *pum* mutants. Given that *nos* and *pum* are required for each other's activity during embryogenesis, they may also act together in germline stem cell maintenance. However, the differences in the Spectrin staining patterns in these two mutants suggests otherwise. If the *nos* phenotype was merely a weak or partial version of the *pum* phenotype then we would expect to see much more reduced aggregates of Spectrin in *pum* mutants. Rather, the differences in the position and appearance of the spectrosomes in *nos* and *pum* mutant germaria suggest different effects of these mutants in germline stem cell function. While *pum* is required for stem cell maintenance, the focus of the *nos* phenotype appears to be on germline cyst development. In addition, while germline stem cells are established in *nos* mutants, these stem cells are abnormal with respect to spectrosome structure, and appear to proliferate slowly and die prematurely. The differences in the phenotypes of *pum* and *nos* mutants are consistent with their overlapping but largely complementary expression patterns.

The differences in the effects of *pum* and *nos* on stem cell maintenance and function suggest that these genes may interact with other partners in the germarium. Recent evidence supports this idea. Arrizabalaga and Lehmann (unpublished data) have shown that mutations in different parts of the Nos protein affect oogenesis and embryonic patterning differently. Analysis of other mutants that cause an early loss of germline from the germarium may provide such partners (Lin and Spradling, 1997; Schüpbach and Wieschaus, 1989, 1991). Specifically, it will be most interesting to look at any such genes that appear to have domains that can interact with RNA, as these may provide alternative partners for Nos in translational repression. One putative candidate for a partner for Nos in the germline is the *orb* gene. *orb* encodes an RNA-binding protein expressed in the same pattern as *nos* in the germarium, and mutants in this gene have similar effects on germline cyst development to *nos* mutants (Christerson and McKearin, 1994; Lantz et al., 1994).

The arrest of germline cyst differentiation at the time when somatic cells normally encapsulate the cysts is a striking feature of the *nos* phenotype. It is possible that *nos* is required for the activation of a factor critical for further cyst development, maybe a cell surface molecule that allows the 16-cell cyst to be recognized by the invaginating somatic prefollicle cells. One reason for the arrest of cyst development may be the paucity of the fusome material spreading between the cystoblasts. Although the function of the fusome is unknown, in another mutant in which fusome structure is lost, *hu li tao sho* (*hts*), cystoblasts fail to undergo the normal four rounds of division and produce egg chambers containing a reduced number of nurse cells and lacking oocytes (Yue and Spradling, 1992). This phenotype is clearly different from that observed in *nos* mutants but indicates that components of the fusome have a role in regulating cystoblast division and differentiation.

nos was isolated as a gene required for abdomen formation in the embryo. However, unlike the anterior determinant *bicoid*, which actively promotes anterior fate, the only requirement for *nos* in embryonic patterning is to repress the translation of maternally deposited *hb* in the posterior. In the absence of maternal *hb*, which appears to have no function itself, *nos* activity is obsolete. We have shown that *nos* activity is essential in the germline at several stages of germline development. Posteriorly localized maternal *nos* is required for germ cell migration, zygotic expression of *nos* is required for the efficient population of the developing ovary by germline cells and *nos* activity is required in the adult germline for early oogenesis. It is possible that these are the original functions of *nos* and that its function in embryonic patterning is more recently evolved. Indeed *nos* homologs in other invertebrates and vertebrates are expressed in the germline, adding weight to the argument for an evolutionarily conserved germline function of this gene (Mosquera et al., 1993; Pilon and Weisblat, 1997).

We are grateful to Laura Dickinson who prepared Nanos antibody stainings of wild-type ovaries. We would like to thank Gustavo Arrizabalaga and Danny Chagnovich for the molecular analysis of the *pumilio* alleles, and past and present members of our laboratory for

reagents and critical suggestions on the manuscript. We are grateful to Claude Desplan for supplying fly strains used in this work and D. Branton for supplying the anti-Spectrin antibody. A. F. is supported by a fellowship from the Human Frontier Science Program and R. L. is an HHMI investigator.

REFERENCES

Barker, D., Wang, C., Moore, J., Dickinson, L. K. and Lehmann, R. (1992). Pumilio is essential for function but not for distribution of the *Drosophila* abdominal determinant Nanos. *Genes Dev.* **6,** 2312-2326.

Chou, T.-B. and Perrimon, N. (1992). Use of a yeast site-specific recombinase to produce female germline chimeras in *Drosophila*. *Genetics* **131,** 643-653.

Christerson, L. B. and McKearin, D. M. (1994). *orb* is required for anteroposterior and dorsoventral patterning during *Drosophila* oogenesis. *Genes Dev.* **8,** 614-628.

Curtis, D., Treiber, K., Feng Tao, Zamore, P., Williamson, J.R. and Lehmann, R. (1997). A CCHC metal-binding domain in Nanos is essential for translational regulation. *EMBO J.* **16** 834-843.

Dahanukar, A. and Wharton, R. (1996). The Nanos gradient in *Drosophila* embryos is generated by translational regulation. *Genes Dev.* **10,** 2610-2620.

Dalby, B. and Glover, D. M. (1993). Discrete sequence elements control posterior pole accumulation and translational repression of maternal *cyclin B* RNA in *Drosophila*. *EMBO J.* **12,** 1219-1227.

Eldon, E. D. and Pirrotta, V. (1991). Interactions of the *Drosophila* gap gene *giant* with maternal and zygotic pattern forming genes. *Development* **111,** 367-378.

Forbes, A. J., Lin, H., Ingham, P. W. and Spradling, A. C. (1996a). *hedgehog* is required for the proliferation and specification of ovarian somatic cells prior to egg chamber formation in *Drosophila*. *Development* **122,** 1125-1135.

Forbes, A. J., Lin, H., Ingham, P. U. and Spradling, A. C. (1996b). The role of segment polarity genes during early oogenesis in *Drosophila*. *Development* **122,** 3283-3294.

Gavis, E. R. and Lehmann, R. (1994). Translational regulation of *nanos* by RNA localization. *Nature* **369,** 315-318.

Gavis, E. R., Lunsford, L., Bergsten, S.E and Lehmann, R. (1996). A conserved 90 nucleotide element mediates translational repression of *nanos* RNA. *Development.* **122**, 2791-2800

Godt, D. and Laski, F. (1995). Mechanisms of cell rearrangement and cell recruitment in *Drosophila* ovary morphogenesis and the requirement of *bric-a-brac*. *Development* **121,** 173-187.

Hülskamp, M., Schröder, C., Pfeifle, C., Jäckle, H. and Tautz, D. (1989) Posterior segmentation of the *Drosophila* embryo in the absence of a maternal posterior organizer gene. *Nature*. **338**, 629-632

Hülskamp, M., Lukowitz, W., Beermann, A., Glaser, G. and Tautz, D. (1994). Differential regulation of target genes by different alleles of the segmentation gene *hunchback* in *Drosophila*. *Genetics* **138**.

Ingham, P. and Forbes, Z. (1993). Microinjection into *Drosophila* embryos. In *Essential Developmental Biology: a Practical Approach.* (ed. C. Stern and P. Holland). Oxford University Press.

Irish, V., Lehmann, R. and Akam, M. (1989). The *Drosophila* posterior-group gene *nanos* functions by repressing *hunchback* activity. *Nature* **338,** 646-648.

King, R. C. (1970). *Ovarian Development in Drosophila melanogaster.* New York: Academic Press.

Kobayashi, S., Yamada, M., Asaoka, M. and Kitamura, T. (1996). Essential role of the posterior morphogen *nanos* for germline development in *Drosophila*. *Nature* **380,** 708-711.

Lantz, V., Chang, J. S., Horabin, J. I., Bopp, D. and Schedl, P. (1994). The *Drosophila orb* RNA-binding protein is required for the formation of the egg chamber and establishment of polarity. *Genes Dev.* **8,** 598-613.

Lehmann, R. and Nüsslein-Volhard, C. (1987). *hunchback*, a gene required for segmentation of an anterior and posterior region of the *Drosophila* embryo. *Dev. Biol.* **119,** 402-417.

Lin, H. and Spradling, A. C. (1993). Germline stem cell division and egg chamber development in transplanted *Drosophila* germaria. *Dev. Biol.* **159,** 140-152.

Lin, H., Yue, L. and Spradling, A. C. (1994). The *Drosophila* fusome, a germline-specific organelle, contains membrane skeletal proteins and functions in cyst formation. *Development* **120,** 947-956.

Lin, H. and Spradling, A. (1997). A novel group of *pumilio* mutations affects the asymmetric division of germline stem cells in the *Drosophila* ovary. *Development* **124,** 2463-2476.

Macdonald, P. M. (1992). The *Drosophila pumilio* gene: an unusually long transcription unit and an unusual protein. *Development* **114,** 221-232.

Margolis, J. and Spradling, A. (1995). Identification and behavior of epithelial stem cells in the *Drosophila* ovary. *Development* **121**, 3797-3807.

Moore, L., Broiher, H. T., Van Doren, M., Lunsford, L. and Lehmann, R. (1998). Identification of genes controlling germ cell migration and embryonic gonad formation in *Drosophila*. *Development* **125**, 667-678.

Mosquera, L., Forristall, C., Zhou, Y. and King, M. L. (1993). A mRNA localized to the vegetal cortex of *Xenopus* oocytes encodes a protein with a *nanos*-like zinc finger domain. *Development* **117,** 377-386.

Muller, H.J. (1932). On the nature and causes of gene mutations. *Proc. of 6th Intl. Congress of Genetics.* **1,** 213-231

Murata, Y. and Wharton, R. P. (1995). Binding of Pumilio to maternal *hunchback* mRNA is required for posterior patterning in *Drosophila* embryos. *Cell* **80**, 747-756

Pilon, M. and Weisblat, A. (1997). A *nanos* homolog in leech. *Development* **124,** 1771-1780.

Rongo, C. and Lehmann, R. (1996). Regulated synthesis, transport and assembly of *Drosophila* germ plasm. *Trends Genetics* **12,** 102-109

Schüpbach, T. and Wieschaus, E. (1989). Female sterile mutations on the second chromosome of *Drosophila melanogaster*. I. Maternal effect mutations. *Genetics* **121,** 101-117.

Schüpbach, T. and Wieschaus, E. (1991). Female sterile mutations on the second chromosome of *Drosophila melanogaster*. II. Mutations blocking oogenesis or altering egg morphology. *Genetics* **129,** 1119-1136.

Smibert, C., Wilson, J., Kerr, K. and Macdonald, P. M. (1996). Smaug protein represses translation of unlocalized *nanos* mRNA in the *Drosophila* embryo. *Genes Dev.* **10,** 2600-2609.

Spradling, A. C. (1993). Developmental genetics of oogenesis. In *The Development of Drosophila melanogaster* (ed. M. Bate and A. Martinez Arias), Vol. I, pp. 1-70. Cold Spring Harbor: Cold Spring Harbor Press.

St Johnston, D. and Nüsslein-Volhard, C. (1992). The origin of pattern and polarity in the *Drosophila* embryo. *Cell* **68,** 201-219.

Struhl, G. (1989). Differing strategies for organizing anterior and posterior body pattern in *Drosophila* embryos. *Nature.* **338,** 741-747.

Tautz, D. and Pfeifle, C. (1989). A non-radioactive in situ hybridization method for the localization of specific RNAs in *Drosophila* embryos reveals translational control of the segmentation gene *hunchback*. *Chromosoma* **98,** 81-85.

Wang, C., Dickinson, L. K. and Lehmann, R. (1994). The genetics of *nanos* localization in *Drosophila*. *Developmental Dynamics* **199,** 103-115.

Wharton, R. P. and Struhl, G. (1991). RNA regulatory elements mediate control of *Drosophila* body pattern by the posterior morphogen *nanos*. *Cell* **67,** 955-967.

Whitfield, W. G. F., González, C., Sánchez-Herrero, E. and Glover, D. M. (1989). Transcripts of one of two *Drosophila* cyclin genes become localized in pole cells during embryogenesis. *Nature* **338,** 337-340.

Williamson, A. and Lehmann, R. (1996). Germ cell development in *Drosophila*. *Ann. Rev. of Cell and Dev. Biol*. **12**, 365-391.

Yue, L. and Spradling, A. C. (1992). *hu-li tai shao*, a gene required for ring canal formation during *Drosophila* oogenesis, encodes a homolog of adducin. *Genes Dev.* **6,** 2443-2454.

Zamore, P., Williamson, A. and Lehmann, R. (1997). The Pumilio protein binds RNA through a domain that defines a new class of RNA-binding proteins. *RNA.* **3**, 1421-1433.

Zhang, N., Zhang, J., Cheng, Y. and Howard, K. (1996). Identification and genetic analysis of *wunen*, a gene guiding *Drosophila melanogaster* germ cell migration. *Genetics* **143,** 1231-1241.