Invertebrate Reproduction and Development, 39:1 (2001) 21–35
Balaban, Philadelphia/Rehovot
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Poached egg, a gene required in the soma to maintain germ cell viability in *Drosophila* females

PHU V. TRAN, JANETTE PETTUS and ROD N. NAGOSHI*
Department of Biological Sciences, Biology Building, University of Iowa, Iowa City, IA 52242, USA
Tel. +1 (319) 335-1076; Fax +1 (319) 335-1069; email: rodney-nagoshi@uiowa.edu

Received 28 August 2000; Accepted 4 January 2001

Summary

In *Drosophila*, extensive interactions between the soma and germline are required for oogenesis. Interactions between the somatic ovary and follicle cells influence the differentiation and organization of the egg chamber. There is also evidence that the soma is needed to maintain germline viability. Little is known about the nature of these interactions or their genetic components. In this paper we identified a gene required in the soma for germline viability. Mutations in *poached egg* result in the induction of apoptosis in the nurse cells of stage 7 and later egg chambers. If oogenesis is arrested prior to vitellogenesis, *poached egg*-induced apoptosis does not occur. This indicates that *poached egg* function is dependent on some stage-specific process which coincides with the onset of vitellogenesis. We describe the genetic analysis of this newly identified gene, including the generation of new alleles and genetic interactions with the yolk protein complex.

Key words: Apoptosis, germline, oogenesis, gametogenesis

Introduction

The maturation of the *Drosophila* egg chamber is subdivided into 14 stages, each distinguished by a characteristic set of morphologies (see Spradling, 1993). Initially, a germline stem cell undergoes a set of four incomplete mitotic divisions to form a 16-cell syncytium interconnected by cytoplasmic ring canals, which is then surrounded by somatically derived follicle cells to form the nascent egg chamber. One germ cell differentiates as the oocyte, while the remaining 15 become the nurse cells which act to support oocyte development. Vitellogenesis begins at stage 7 with the deposition of yolk protein into the oocyte. This results in the progressive expansion of the oocyte, which will ultimately fill the entire chamber.

By oogenic stage 10, the oocyte extends to almost 50% of the egg length. At this time, the nurse cells undergo the process of "dumping", during which the bulk of their cytoplasmic contents are rapidly transported to the oocyte. The subsequent increase in oocyte volume displaces the depleted nurse cells to the anterior tip of the chamber (stages 11–12) where they then undergo rapid degeneration. This death has apoptotic characteristics, including nuclear condensation and DNA fragmentation, but does not require the germline expression of the known *Drosophila* programmed cell death effectors, *reaper*, *grim*, or *head involution defective* (Foley and Cooley, 1998). In addition, mutations in the gene (*dcp-1*) encoding for *Drosophila* caspase, a protease generally required for

*Corresponding author.

apoptotic death, have been identified and their ovarian phenotype examined (McCall and Steller, 1998; Song et al., 1997). Although chambers mutant for *dcp-1* failed to undergo dumping at stage 10, the nurse cells still underwent (albeit delayed) apoptotic-like degeneration (McCall and Steller, 1998). Therefore, the mechanism by which nurse cells normally die in oogenesis is not understood.

There are also sporadic occasions of spontaneous egg chamber degeneration in wild-type ovaries. In this case, the affected chambers contain nurse cells with condensed nuclei, vesicularization of nuclear material, and membrane blebbing, all suggestive of an apoptotic mechanism (Giorgi and Deri, 1976). It was hypothesized that egg chambers spontaneously experiencing defective development are aborted through a programmed death process. Consistent with this suggestion, we found that environmental stress and age, which should make developmental errors more likely, increase the frequency of degenerating chambers in wild-type ovaries (Chao and Nagoshi, 1999). The mechanism for this elimination of defective chambers is not known, though recent studies suggest the involvement of an ecdysone-dependent pathway (Buszczak et al., 1999).

The basis for the stage specificity of these germline death phenomena is not known. Of potential relevance is the observation that the *DIAP1* and *DIAP2* (*Drosophila* homologs of baculovirus inhibitors of apoptosis) genes are expressed in nurse cells throughout most of oogenesis, but are down-regulated during stages 7 and 8 (Foley and Cooley, 1998). Since both genes are capable of suppressing programmed cell death in the eye (Hay et al., 1995), this germline pattern of expression potentially provides a window of opportunity for the activation of apoptotic death mechanisms during mid-stages of oogenesis. If correct, then maintaining germline viability would depend on the active suppression of an already present cell death mechanism.

Oogenic stages 7–8 coincide with the onset of yolk deposition in the oocyte. Normal vitellogenesis is dependent on the expression of the three yolk protein genes in Drosophila, *Yp1*, *Yp2*, and *Yp3* (Bownes, 1979). Most yolk protein is expressed in the adult female fat body where it is secreted into the hemolymph, then taken up by the oocyte through interfollicular spaces in the follicle layer. Significant yolk protein is also produced by the follicle cells and can be directly translocated to the oocyte surface (reviewed in Bownes, 1994).

Although no single yolk protein is essential for oogenesis, reductions in the dosage of one or all proteins can result in reduced fertility (Bownes et al., 1991). This phenotype is associated with both a decrease in egg production and a decline in hatching rates, the latter indicating defective egg chamber structure. In addition, certain alleles of the yolk protein genes are capable of causing complete female sterility by producing a defective peptide that interferes with oogenesis (Butterworth et al., 1991; Butterworth et al., 1992). The $Yp1^{ts1}$ allele is a single base change causing an amino acid substi-tution. Females homozygous for $Yp1^{ts1}$ are sterile at all temperatures while heterozygotes and hemizygotes are sterile at 29°C and partially fertile at 18–22°C. The mutant protein is expressed in fat bodies and secreted, but precipitate as globules or crystalline fibers in the fat body. Similar precipitates are observed in vitellogenic egg chambers both in the space between the plasmalemma and vitelline membrane and in the membrane itself (Butterworth et al., 1992). These disruptions are the likely cause of the aberrantly flaccid chambers associated with $Yp1^{ts1}$ sterility.

In this paper we present the genetic characterization of a newly identified gene required to maintain nurse cell viability. Mutations in *poached egg* (*peg*) cause the apoptotic degeneration of the female germline during vitellogenic stages of oogenesis. The *peg* mutations map near the *Yp1–Yp2* complex and show dominant interactions with $Yp1^{ts1}$. However, the examination of chromosomal rearrangements indicate that *peg* is physically separable from the yolk protein loci, and so represents an independent gene. We demonstrate that the action of *peg* is specific to late stages of oogenesis, affecting the organization of the microtubule cytoskeleton, as well as the induction of apoptotic death. We further show that although *peg* mutations seem to specifically affect the germline, *peg* expression is only required in the soma to maintain normal oogenesis. Therefore, *peg* may be part of a previously unknown somatic process required to maintain germline viability.

Material and Methods

Fly strains and crosses

Unless otherwise noted, alleles and chromosomes used are described in Lindsley and Zimm (1992). peg^1 and hts^1 double mutants were derived from the cross, w^{1118} peg^1/*FMO*; *cn* hts^1/*CyO* X w^{1118} peg^1/*Y*; *cn* hts^1/*CyO*. Flies doubly mutant for peg^1 and *otu* were derived from the following crosses: (1) *y cv* otu^4

peg^{1}*/FMO* X *y cv otu*^{4} *peg*^{1}*/Y*, and *y cv otu*^{5} *peg*^{1}*/FMO* X *y cv otu*^{5} *peg*^{1}*/Y*.

Mutageneses

w1118/Y males were fed 25 mM EMS in 5% sucrose for 24 h using standard protocols (Ashburner, 1989). The mutagenized (*w1118*/Y*) males were mated en masse to *FMO/ClB* females and the *w1118*/FMO* and *w1118*/ClB* female progeny were individually mated to *FMO/Y* males. The progeny from each pair mating was examined for the presence of an X-linked lethal by the absence of *B+* (*w1118*/Y*) males. Pair matings with viable mutagenized X-chromosomes were tested for the presence of female-sterile lesions. The mutagenized chromosomes were made homozygous and the resulting females were tested for fertility. From 1821 mutagenized chromosomes we obtained 756 (41.5%) X-linked lethals and 66 (3.6%) X-linked female-steriles. The *peg*^{1} allele was recovered from this latter group.

New alleles of *peg* were obtained by gamma-irradiation. *w*^{1118} *peg*^{1}*/Y* males were irradiated with 4000R of gamma-irradiation (mutagenized chromosomes are designated *peg*^{1}*). These were mated in small groups (20 females to 10 males) to *Yp1*^{ts1}*/FMO* at 22°C. Both *peg*^{1}*/Yp1*^{ts1} and *peg*^{1}*/Df(1)C52* are sterile at 22°C, while *Yp1*^{ts1}/+ or *Yp1*^{ts1}/deletion is semi-fertile. Therefore, most *peg*^{1}*/*Yp1*^{ts1} daughters are sterile. However, if *peg*^{1} expression is eliminated by a second mutation, then heterozygotes with *Yp1*^{ts1} should produce progeny. In four separate vials, progeny were obtained. In each case, male progeny were crossed to *Yp1*^{ts1}*/FMO* females. The *peg*^{1}*/*Yp1*^{ts1} daughters were retested for fertility at 22°C, while the sibling *peg*^{1}*/*FMO* daughters were crossed to *FMO/Y* males to created a stock. The four *peg* alleles were again fertile over *Yp1*^{ts1} at 22°C and were designated *peg*$^{2\text{-}5}$.

Examination of DNA fragmentation

Terminal deoxynucleotide transferase (TdT)-mediated dUTP-biotin nick end-labeling (TUNEL) was performed as previously described (Gavrieli et al., 1992; Chao and Nagoshi, 1999).

DNA fragmentation was also examined by gel electrophoresis. Ovaries from approximately 100 flies were dissected in PBS and transferred into 0.5 ml of DNA lysis buffer (100 mM Tris-pH 8.0, 5 mM EDTA, 0.2% SDS, and 200 mM NaCl) containing 100 µg of Proteinase K. Nuclei were pelleted by centrifugation at 10K rpm for 5 min. DNA was purified by phenol/chloroform extraction and ethanol precipitation. RNAs were removed by adding 0.04 g/L RNAseA and incubating at 37°C for 2 h. DNA was purified by phenol/chloroform extraction and ethanol precipitation. Approximately 10 µg of isolated DNA was electrophoresed overnight on a 1.5% agarose gel at 2.5 V/cm and 4°C, then stained with ethidium bromide using standard procedures (Maniatis et al., 1982).

Morphological analyses of gonads

The morphology of mutant gonads was examined by either Feulgen or 4',6-diamidine-2-phenylindole (DAPI) staining. Flies were cultured at 25°C. Female flies of the appropriate genotypes were aged 7–8 days after eclosion at 25°C. The ovaries were dissected and stained by Feulgen reaction (Galigher and Kozloff, 1971) or DAPI as previously described (Geyer et al., 1993).

ß-galactosidase staining

The *kin-lacZ* construct has been previously described (Clark et al., 1994). The cross *w peg*^{1}*/FMO*; *kin-lacZ/CyO X w peg*^{1}*/Y*; *kin-lacZ/CyO* was used to produce *peg*^{1} mutants containing *kin-lacZ*. These were then examined for the pattern of ß-galactosidase expression in the ovaries as previously described (Hinson and Nagoshi, 1999).

Polytene chromosomes analysis

peg mutants and relevant rearrangements were crossed to *w*^{1118} flies and cultured at 18°C. Polytene chromosome preparations from isolated third instar salivary glands were performed by standard methods (Ashburner, 1989). Preparation of polytene chromosomes for *in situ* hybridization was performed as previously described (Lim, 1993). Digoxygenin-labeled probes were synthesized by the random priming method according to manufacturer's instructions (Roche/Boehringer Mannheim).

Germline clonal analysis

Germline clones were produced by the dominant female-sterile procedure (Perrimon and Gans, 1983). *ovoD1* (or *fs(1)K1237*) blocks oogenesis when present in one copy in the germline stem cells. The progeny from the mating of *sn*^{3} *peg*^{1}*/FM0* females to *ovoD1 v24/Y* males were irradiated with 1000 Rads from a 137Cs gamma source at 44–52 h post-oviposition to induce mitotic recombination in the germline

(Wieschaus and Szbad, 1979). Irradiated females of the genotype *sn³ ovo+ peg¹ /ovoD1 peg+ v24* were tested for fertility by matings with *sn³ peg¹/Y* males. Clones resulting from recombination events proximal to *v* must be *ovo+ peg1* mutant, producing eggs of the genotype *sn³ ovo+ peg1*. These proximal clones were identified by progeny that were *sn³* and *peg¹* mutant. As a control, larvae from the cross of *y¹ cv fs(1)Yb¹ v²⁴ f/FMO* to *ovo^D1 v²⁴/Y* were irradiated as above. *fs(1)Yb¹* is a soma-dependent female-sterile mutation and so should generate fertile germline clones (Johnson et al., 1995).

Protein extracts and Western blot analysis

Protein samples were isolated from 20 8–10-day-old adult flies by dounce homogenization in 200 µl of protein extraction buffer (145 mM NaCl, 4 mM KCl, 1 mM EGTA, 1 mM $MgSO_4$, 10 mM Na phosphate buffer, pH=7). The extract was incubated on ice for 30 min and cellular debris pelleted by spinning in a microcentrifudge for 10 min at 4°C. The supernatant was mixed 1:1 with 2X-SDS-PAGE loading buffer and the proteins denatured by heating 5 min at 95°C; 20 µl aliquots were loaded per lane on two 10% polyacrylamide gels, with Bio-Rad Precision Protein Standards as molecular weight markers. One gel was used for the Western blot and the other stained with Coomasie Blue to control for loading. The gels were simultaneously run for 1 h at 100 V in cold 1× electrophoresis buffer diluted from a 10× stock solution (30.2 g Tris, 144 g glycine, 10 g SDS, ddH_2O to liter). The gel was equilibrated in transfer buffer (2.5% Tris, 19% glycine, 15% MeOH, 0.01% SDS) for half an hour at room temperature, then electroblotted to PVDF membrane in ice-cold transfer buffer at 100 V for 1 h in a Bio-Rad Mini Trans-Blot apparatus. The membrane was rinsed for 10 min in sterile ddH_2O, then blocked overnight at 4°C with gentle rocking in a TTBS+3% casein blocking solution (TTBS= 100 mM Tris, pH=7.5, 0.9% NaCl, 0.1% Tween20). The polyclonal rabbit anti-YP primary antibody (gift from M. Bownes) was diluted 1:250 in TTBS+3% casein and pre-absorbed against 0.6 mg acetone-dried powdered adult male tissue for 45 min at room temperature to reduce non-specific background. The pre-blocked filter was washed 3× for 15 min with TTBS and incubated with the anti-YP primary antibody for 2 h. After three 15-min washes with TTBS, the blot was incubated for 1 h with goat anti-rabbit IgG-AP conjugate (Sigma-Aldrich) diluted 1:10,000 in TTBS+3% casein. Molecular weight standards were detected by addition of Bio-Rad's Precision StrepTactin-AP conjugate at a 1:7500 dilution. The filter was washed 3× for 15 min with TTBS followed by two 10-min washes with TBS before color detection in Sigma Fast BCIP/NBT buffer.

Results

Isolation and genetic mapping of the peg¹ *mutation*

The original mutant allele, initially called *fs(1)513¹*, was isolated from an EMS mutagenesis designed to identify male-viable, female-sterile mutations on the X-chromosome. *fs(1)513¹* is recessive, causing complete sterility in homozygous females raised at 18°C, 25°C, or 29°C. Male viability and fertility are unaffected. Dissection of mutant female ovaries showed a few mature eggs amidst degenerating egg follicles. In comparison, *fs(1)513¹* mutant male gonads displayed no obvious morphological defects. Based on this phenotype, we renamed the gene *poached egg* (*peg*).

The *peg¹* mutation was mapped by recombination to approximately three centimorgans proximal from *lozenge (lz)*. More detailed mapping was performed

Table 1. Mutations and deletions tested for complementation to *Yp1^ts1* and *peg¹*

Genotype	Cytology	Fertile over *Yp1^ts1* at 22° or 25°C?	Fertile over *peg¹* at 22° or 25°C?
Df(1)C52	8E4; 9C1-4[a]	No	No
Df(1)N110	9B3-4; 9D1-2[ab]	Yes	No
Df(1)HK^KSW5	9A2-3; 9B8-11[bc]	Yes	No
Df(1)v^L15	9B1-2; 10A1-2[a,b]	Yes	Yes
Df(1)Hk^JE3A2	9B3-4; 9B7-8[bc]	Yes	Yes
Tp(1;2)v^+75d	9A2; 10C2[a]	No	Yes
Yp^ts1	9A2-4[a]	No	No
peg¹	cytologically normal[b]	No	No
peg²	Inversion with 9A breakpoint[b]	Yes[d]	No
peg³	Inversion with 9A breakpoint[b]	Yes[d]	No
peg⁴	Rearrangement within 9A[b]	Yes[d]	No
peg⁵	Inversion with 9A breakpoint[b]	Yes[d]	No

[a]As listed in Lindsley and Zimm (1992).
[b]Determined or confirmed by polytene chromosome analysis in this study (data not shown).
[c]As listed in Schlimgen (1991).
[d]Fertility is less than 10% of wild type, but is reproducible.

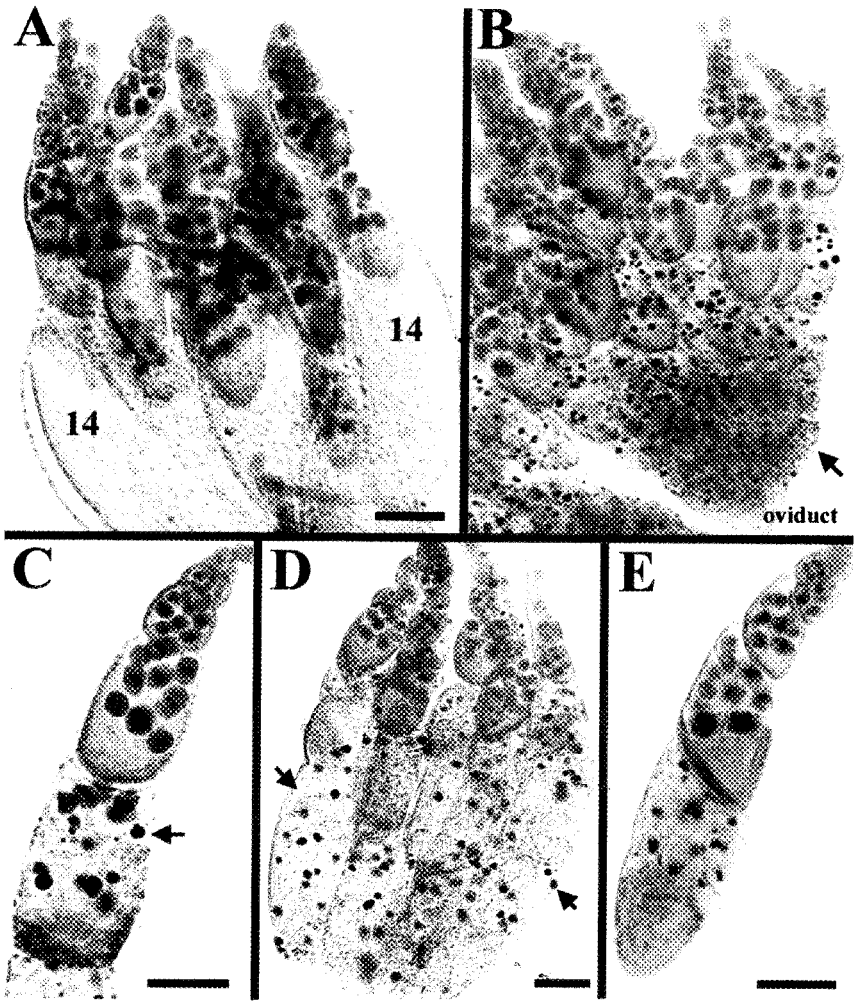

Fig. 1. *peg* mutant phenotypes. Ovaries were dissected from 7–10-day-old females and stained by Feulgen. Nuclei stain dark. A. Portion of a wild-type ovary lobe showing several oogenic stages. Mature chambers at stage 14 (14) are filled with yolk and lack nurse cells. B. Typical peg^1 mutant ovary lobe contains normal chambers until about stage 7–9. More mature chambers contain degenerating nurse cells. Arrow points to disorganized mass of debris near oviduct. C. Single peg^1 mutant ovariole displaying transition from normal stage 8 chamber to degenerating chamber (arrow). D. Cluster of peg^1 mutant ovarioles with more mature chambers (arrows) containing dispersed condensed nuclear material. E. Single $peg^1/Yp1^{ts1}$ mutant ovariole displaying a similar phenotype as peg^1 mutants. Bars = 100 microns. Plates A and B are at the same magnification.

using existing deletions with breakpoints in the region (Table 1). The peg^1 allele failed to complement either *Df(1)C52* (cytological position 8E; 10C5) or *Df(1)N110* (9B3-4; 9D), indicating that at least portions of the gene are located within the 8E through 9D interval. The localization was further refined to the region between 9A to 9B, based on the complementation with $Df(1)Hk^{JE3A2}$ and $Df(1)v^{J.15}$ (deletions of 9B3-4; 9B7-8 and 9B1-2; 10A1-2, respectively) and the ability of the second chromosome duplication $Tp(1;2)v^{+75d}$ to rescue peg^1 homozygotes (Table 1). The latter observation defines polytene chromosome region 9A2 as the distal limit of the *peg* gene.

Phenotype of the peg mutations

In mutant peg^1 ovaries, the developing eggs undergo a striking stage-specific degeneration. *Drosophila* ovaries are made up of a cluster of tubular ovarioles. Oogenesis begins at the apical tip of the ovariole, the germarium, with the developing chambers moving distally to the oviduct. The result is a linear array of progressively maturing eggs. Previtellogenic chambers are relatively small and consist primarily of nurse cells (noted by their large, polyploid nuclei), while mature chambers contain mostly yolk (Fig. 1A). Yolk accumulation in the oocyte becomes apparent at oogenic stage 7, marking the onset of the vitellogenic

stages. In *peg*^{1} mutant ovaries, the egg chambers display wildtype development up to stage 7, with normal nurse cell and oocyte morphology and numbers (Fig. 1B). However, subsequent stages show striking abnormalities, with the nurse cell nuclei becoming misshapen, fragmented, then highly condensed (Fig. 1B,C). The nuclear aberrations are associated with a general disruption of germ cell integrity and egg chamber organization, as seen by the loss of clear boundaries between individual nurse cells and the oocyte. Ultimately, the egg disintegrates, with debris collecting in a disorganized mass near the oviduct (arrow, Fig. 1B).

There is some variability in the severity of the *peg*^{1} mutant phenotype, depending on temperature and culture conditions. At 18–22°C, more mature egg chambers are common than seen at 25°C, with development sometimes progressing as far as stage 14 (Fig. 1D). However, even under the most optimum conditions, mutants remain completely female-sterile with condensed, pycnotic nuclei often present in the more mature chambers.

Genetic interactions with $\mathbf{Yp1^{ts1}}$

Located in the vicinity of the *peg*^{1} mutation is the *Yp1-Yp2* yolk protein complex, which maps to cytological region 9A2-4 (Lindsley and Zimm, 1992). The absence of wild-type Yp1 or Yp2 protein reduces female fecundity (number of eggs laid), though viable egg chambers are still produced. In addition, there exists a semi-dominant, temperature-sensitive allele of *Yp1* that can completely disrupt oogenesis. Females homozygous for *Yp1*^{ts1} are sterile at all temperatures, producing flaccid eggs that incorporate reduced levels of yolk protein (Giorgi and Postlethwait, 1985). *Yp1*^{ts1}/+ heterozygotes and *Yp1*^{ts1}*/Df(1)C52* hemizygotes are partially fertile at 18°C and completely female-sterile at 29°C (Bownes and Hodson, 1980; Postlethwait and Shirk, 1981). At 25°C these genotypes differ in their phenotypic severity, as *Yp1*^{ts1}/+ females are partially fertile while *Yp1*^{ts1}*/Df(1)C52* remain sterile (Table 1). The *Yp1*^{ts1} mutant phenotype is not associated with the induction of germ cell death at any temperature, and so differs from *peg*^{1} homozygotes.

Despite these phenotypic distinctions, female flies transheterozygous for *Yp1*^{ts1} and *peg*^{1} (*Yp1*^{ts1} *peg*$^{+}$*/Yp1*$^{+}$ *peg*^{1}) were sterile at 22–25°C and partially fertile at 18°C. The ovaries produced at 25°C resembled those seen in *peg*^{1} mutants, with many vitellogenic egg chambers containing degenerating nurse cells (Fig. 1E). This lack of complementation suggests that *peg*^{1} might be a lesion in one of the yolk protein genes. However, the deletions *Df(1)Hk*^{KSW5} and *Df(1)N110* are both mutant for *peg* activity, yet each can complement *Yp1*^{ts1} at 25°C (Table 1). This genetic indication that neither deletion removes the *Yp1* complex was supported by *in situ* analyses of salivary polytene chromosomes from flies homozygous for *Df(1)Hk*^{KSW5} and carrying a autosomal duplication of the region (Fig. 2A). *Yp1*-specific probes hybridize to the appropriate site on the X-chromosome, indicating that this deletion did not remove *Yp1*. Interestingly, *Yp1* sequences are present in the *Tp(1;2)v*$^{+75d}$ insertional duplication (Fig. 2B), even though this duplication cannot complement *Yp1*^{ts1} homozygotes or hemizygotes (Table 1). This suggests that the *Yp1* sequences present are not fully functional, yet this duplication fully complements *peg*^{1} homozygotes to fertility (Table 1). Finally, we examined the levels of yolk protein in females wildtype or mutant for peg (Fig. 3). We could discern no significant difference in expression levels. Based on these data, we conclude that *peg* and *Yp1* represent different genes, mutant alleles of which display dominant interactions.

Mutagenesis and further genetic mapping of the peg *locus*

Unlike *peg*^{1}, the *peg* mutant deletion *Df(1)Hk*^{KSW5} complements *Yp1*^{ts1} at 22–25°C. This suggests that the *peg*^{1} allele produces an abnormal function capable of deleteriously interacting with the mutant *Yp1*^{ts1} yolk protein. If correct, it should be possible to revert the sterility associated with *Yp1*^{ts1} *peg*$^{+}$*/Yp1*$^{+}$ *peg*^{1} transheterozygotes by inducing a secondary mutation that prevents the expression of the *peg*^{1} product. To inactivate the *peg*^{1} gene, we performed a gamma ray mutagenesis of the *Yp1*$^{+}$ *peg*^{1} chromosome and selected for the loss of genetic interactions with *Yp1*^{ts1}. *Yp1*$^{+}$ *peg*^{1}/Y males were irradiated and crossed to *Yp1*^{ts1} *peg*$^{+}$*/FM0* females. *Yp1*^{ts1} *peg*$^{+}$*/Yp1*$^{+}$ *peg*^{1}* daughters (* indicates a mutagenized chromosome) were collected and mated en masse to *FM0/Y* males at 22°C, a temperature where *Yp1*^{ts1} *peg*$^{+}$*/Yp1*$^{+}$ *peg*^{1} females are sterile while *Yp1*^{ts1}/+ heterozygotes are fertile. Hence, mutations inactivating *peg*^{1} expression should restore fertility to the *Yp*^{ts1} *peg*$^{+}$*/Yp*$^{+}$ *peg*^{1}* females. In a screen of 18,000 *peg*^{1} chromosomes, four were identified that were partially fertile over *Yp1*^{ts1}. Each has no apparent effects on males but results in complete female-sterility when homozygous. Consistent with these being loss-of-function mutations in the

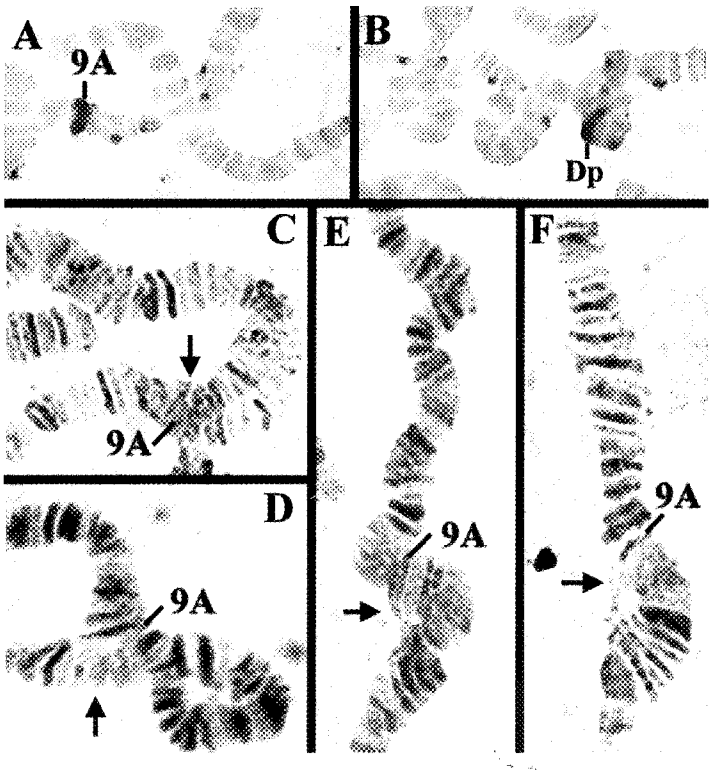

Fig. 2. Mapping of chromosomal rearrangements of the *peg* region by analysis of polytene chromosomes. Polytene chromosomes were isolated from salivary glands of third instar larvae. A. The 9A region of the X-chromosome from a strain carrying one copy of the $Tp(1;2)v^{+75d}$ duplication. The *peg*-containing duplicated X-chromosome region is located at the base of chromosome 2L. Note this is different than previously suggested in Lindsley and Zimm (1992). The preparation was hybridized to a probe specific to *Yp1*. The *Yp1* locus on the X-chromosome is labeled (arrow head). The duplication loop (arrows) is not hybridized by the *Yp1* probe. B. Higher magnification view of the 9A region of the X-chromosome shown in A. C. Higher magnification view of of the $Tp(1;2)v^{+75d}$ duplication shown in A. Unlike the X-chromosome, the duplicated region does not contain the *Yp1* complex. D–G display polytene chromosomes stained to display chromosome banding. In each case, a *peg* mutant chromosome is heterozygous with an unrearranged w^{1118} chromosome. Arrows point to breakpoint in 9A region. D. 9A region from peg^2/w^{1118} genotype. A relatively large inversion loop has formed. E. 9A region from peg^3/w^{1118} genotype. An inversion loop has formed. F. 9A region from peg^4/w^{1118} genotype. A small disruption is consistently seen in the 9A region indicating either a small deletion or inversion. G. 9A region from peg^5/w^{1118} genotype. Homologues partially separate because of what appears to be a small inversion.

peg gene, all four failed to complement the original peg^1 allele and were therefore designated $peg^{2\text{-}5}$. Analysis of polytene chromosomes indicate that peg^2, peg^3, peg^5 are likely to be inversions with one breakpoint in the 9A region (Fig. 2D, E, G), while peg^4 appears to be associated with a small rearrangement within 9A (Fig. 2F). In all the new *peg* alleles, *in situ* studies of polytenes using *Yp*-specific probes indicate that the *Yp* region remains intact in these rearranged chromosomes. This genetic indication was further supported by the ability to amplify *Yp1* and *Yp2* genes by PCR from these *peg* mutant alleles (data not shown).

The newly generated *peg* alleles were tested for their mutant phenotype and interactions with $Yp1^{ts1}$.

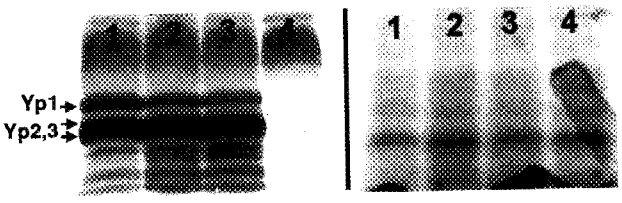

Fig. 3. Analysis of yolk protein expression from whole flies. Extracts from wild-type females (lane 1), peg^1 mutant females (lane 2), peg^2 mutant females (lane 3), and males (lane 4) were loaded unto a 10% linear polyacrylamide gel. A. A polyclonal antibody that recognizes YP1, YP2, YP3 was used in a Western blot. Size standards (not shown) were used to identify the female-specific yolk protein bands. B. Coomasie blue stained gel run in parallel under identical conditions as a control for loading.

Homozygotes of these alleles caused germ cell degeneration in vitellogenic chambers similar to that observed with peg^1, though peg^4 and peg^5 appeared to allow a higher frequency of more mature eggs (data not shown). All four alleles failed to complement $yp1^{ts1}$ at 25°C, displaying a phenotype similar to $peg^1/yp1^{ts1}$ heterozygotes (Table 1).

peg *mutations induce germline apoptosis*

The nuclear aberrations in peg^1 mutant germ cells suggested that the cellular degeneration might be occurring by an apoptotic mechanism. To further examine this possibility we tested peg^1 mutant ovaries for evidence of early DNA fragmentation, a stereotypic characteristic of most programmed cell death phenomena (Wyllie et al., 1984). In the first set of experiments, we used TUNEL analysis to detect DNA fragmentation *in situ* (Gavrieli et al., 1992; White et al., 1994). In wild-type ovaries, TUNEL-labeling in the germline is mostly limited to stages 12–14, when nurse cells undergo their normal degeneration (Foley and Cooley, 1998). In addition, we occasionally found egg chambers that appeared to be spontaneously undergoing DNA fragmentation (Fig. 4A). These TUNEL-positive cells were only found in stage 7 or older chambers. Similar aberrant chambers in otherwise normal ovaries had been identified on the basis of their abnormal morphology, particularly in females undergoing environmental stress (Chao and Nagoshi, 1999; Giorgi and Deri, 1976).

In peg^1 mutants, TUNEL-positive nurse cells were observed in egg chambers beginning at about stage 7–8 and preceded nuclear condensation (vertical arrow, Fig. 4B). The initial appearance of TUNEL-labeling is not uniform within an egg chamber, indicating that DNA fragmentation does not occur simultaneously among the nurse cells in an egg. This observation is noteworthy given the fact that the nurse cells are interconnected by ring canals and so are capable of substantial cytoplasmic exchange. Confirmation of increased DNA fragmentation in peg^1 mutants came from gel electrophoretic examination of genomic DNA. Apoptotic death is associated with the formation of a transient oligonucleosomal "ladder" associated with intermediate stages of chromosomal fragmentation. We found that the peg^1 mutation results in the formation of such a ladder when DNA from isolated ovaries is compared with wild type (Fig. 4C). These observations strongly suggest that the peg^1-associated nurse cell death occurs by an apoptotic process.

Similar phenotypes were observed in the degenerating germ cells in peg^{2-5} homozygotes. These include nuclear condensation and TUNEL-labeling, indicating that apoptotic germ cell death is a general consequence of *peg* loss-of-function mutations.

Microtubule-based transport is disrupted prior to the apoptotic phenotypes

We were interested in determining whether any aberrations in the germline preceded the first observable apoptotic phenotypes. During the early stages of vitellogenic development, microtubules form a network extending from the oocyte to the supporting nurse cells, providing a means by which mRNAs, rRNAs and macromolecules can be transported into the oocyte from the nurse cells (reviewed by Cooley and Theurkauf, 1994). It is likely that major aberrations in germline development will be evident by disruptions in this microtubule transport system. The integrity of microtubule organization can be approximated by the use of the *kin-lacZ* construct, in which the bacterial *lacZ* gene is fused to sequences encoding the mechano-chemical domain of kinesin (Clark et al., 1994). The fusion protein was shown to be initially expressed at about stage 7–8 at the anterior tip of the chamber, subsequently becoming localized to the posterior of the oocyte in a microtubule-dependent manner (Clark et al., 1994) (Fig. 5A,B).

This process is disrupted by the peg^1 mutation. Mutant stage-7 egg chambers displayed multiple ectopic sites of *kin-lacZ* fusion protein accumulation (Fig. 5C). This phenotype occurs prior to the nuclear aberrations, suggesting it to be one of the earlier events affected by *peg* mutations. In this respect, it should be noted that we observed similar disruptions in microtubule-based transport with hypomorphic alleles of the female-sterile genes *ovarian tumor* (*otu*) and *ovo* (Fig. 5D,E). In both mutants, oogenesis is disrupted at about stages 7–10, with no induction of apoptotic germline death (Geyer et al., 1993; King and Riley, 1982; Oliver et al., 1987). Taken together, these observations suggest that the disruption of the microtubule network is not itself sufficient to induce the peg^1 phenotype. This conclusion is consistent with previous studies in which the microtubule network was disrupted by the ectopic application of the microtubule-depolymerizing agent taxol. In these studies, there was no apparent induction of premature egg chamber degeneration (Pokrywka and Stephenson, 1995).

The induction of germline apoptosis is restricted to post-stage 7 chambers

One of the most striking characteristics of the *peg* mutant phenotype is the stage-specificity of the nurse

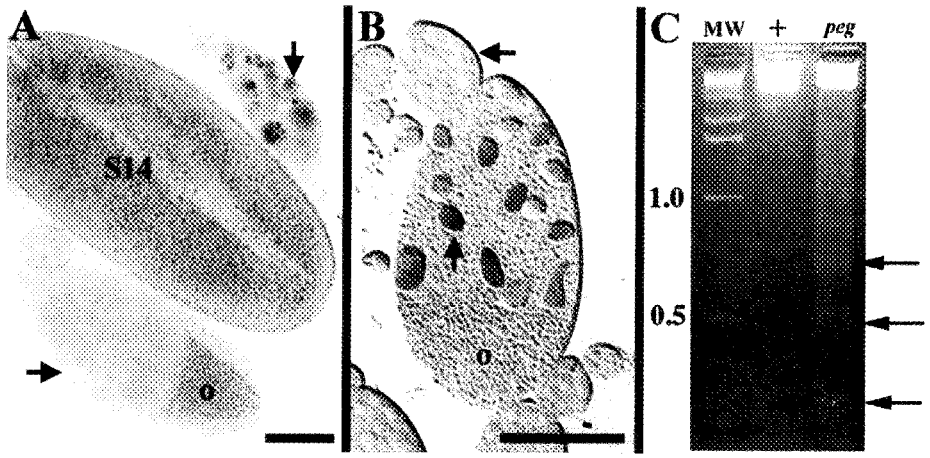

Fig. 4. Examples of apoptotic death in female germ cells. A. In wild-type ovaries egg chambers are occasionally seen that spontaneously degenerate. These display TUNEL-positive nurse cells (dark stain) during stage 7 (vertical arrow). Other chambers do not show evidence of DNA fractionation (horizontal arrow). Yolk in oocyte (o) and mature chamber (S14) shows background staining. B. *peg*^{1} mutant ovariole with previtellogenic (horizontal arrow) and vitellogenic chambers. Stage 9 chamber displays both labeled and unlabeled nuclei. Vertical arrow points to TUNEL-positive nurse cell nucleus. Some nuclei have aberrant shapes. C. Ethidium bromide staining of agarose gel displaying DNA laddering. Genomic DNA from wild type (+) and *peg*^{1} mutant (*peg*) ovaries were isolated and electrophoresed in a 1.5% agarose gel. DNA from mutant ovaries show the formation of oligonucleosomal fragments (arrows), a characteristic of the DNA fragmentation associated with apoptosis. The MW lane contains size standards.

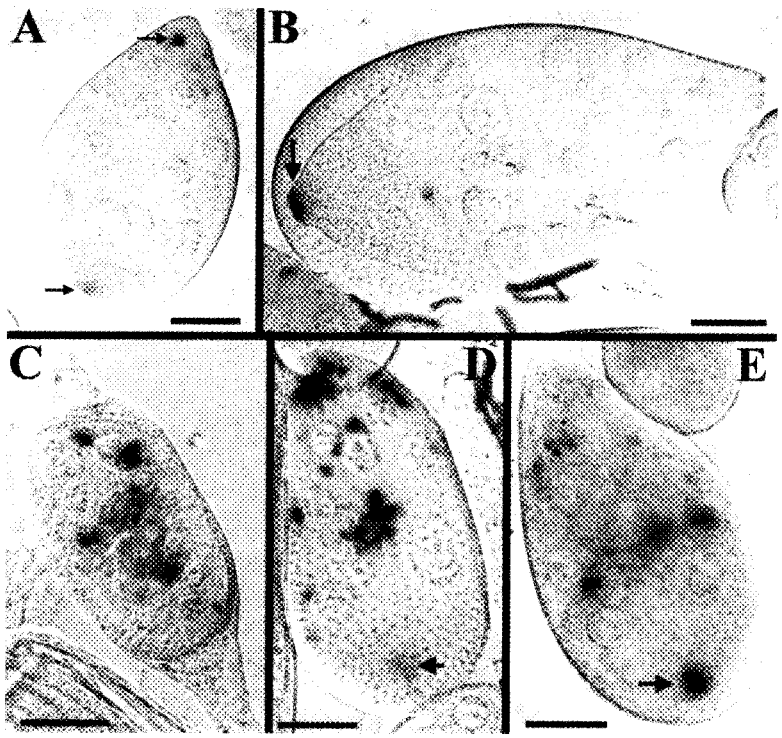

Fig. 5. Expression of *kin-lacZ* in wild-type and *peg* mutant chambers. Ovaries from flies carrying one copy of *kin-lacZ* on the second chromosome were stained for ß-galactosidase activity (stains dark). A. Wild-type stage-7 chamber showing ß-galactosidase expression (arrows) at the anterior tip. Minor expression is seen in the oocyte. B. Wild-type stage9 chamber showing predominant *Kin-lacZ* protein localization in the posterior oocyte (arrow). C. Homozygous w^{1118} *peg*^{1} mutant stage 7–8 chamber with patchy and ectopic *kin-lacZ* expression. D. Homozygous otu^{PD5} *v f* mutant chamber with patchy *kin-lacZ* expression. Some product has localized to the oocyte (arrow). E. Homozygous ovo^{rm1} *v* mutant chamber with ectopic *kin-lacZ* expression. Substantial *Kin-lacZ* product has also localized to the oocyte (arrow). Bars = 50 microns.

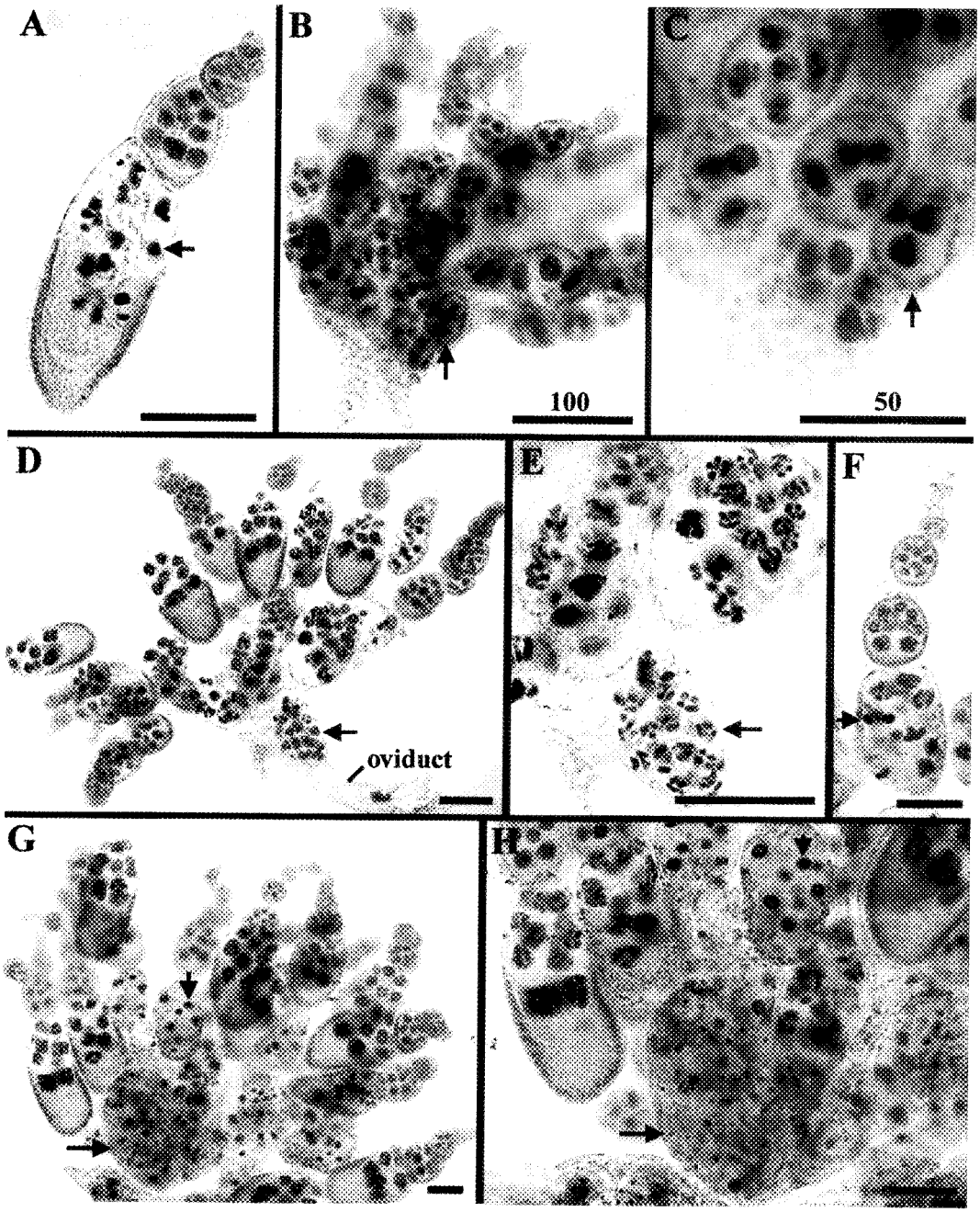

Fig. 6. The effect of *peg* mutations in arrested egg chambers. Nuclei were treated by Feulgen reagent and stained dark. A. Portion of ovariole from *peg*^{1}/*peg*^{1}; *hts*^{1}/+ female derived from the cross w^{1118} *peg*^{1}/*FMO; cn hts*^{1}/*CyO* X w^{1118} *peg*^{1}/*Y; cn hts*^{1}/*CyO*. The typical *peg* mutant phenotype is observed. Arrow points to example of condensed nucleus. B. Ovary lobe from *peg*^{1}/*peg*^{1}; *hts*^{1}/*hts*^{1} sibling from same cross as A. Chambers are arrested at about stage 4, and no apoptotic nurse cells are observed. Chambers typically have fewer nurse cells than normal (arrow). C. Higher magnification view of portion of ovary in B. Nurse cell nuclei appear normal (arrow). D. *otu*^{4} *peg*^{1}/*otu*^{4} *peg*^{1} mutant ovary do not show extensive nurse cell degeneration. The *otu*^{4} allele arrests oogenesis prior to stage 8, which seems to prevent the *peg* mutation from inducing nurse cell apoptosis in most chambers, even those nearest the oviduct (arrow). Note that nurse cell nuclei contain polytene chromosomes (discontinuous staining). E. Higher magnification view of *otu*^{4} *peg*^{1}/*otu*^{4} *peg*^{1} ovary near the oviduct. Most nuclei are not condensed. F. *otu*^{4}*peg*^{1}/*otu*^{4}*peg*^{1} ovariole showing egg chamber with degenerating nurse cells. Presumably this represents a chamber that had reached a stage sensitive to the *peg* mutation. Note that only a subset of nuclei are condensed (arrow). G. Ovary of the genotype *otu*^{5}*peg*^{1}/*otu*^{5}*peg*^{1}. This *otu* allele blocks oogenesis later than *otu*^{4}. These ovaries typically show extensive nurse cell degeneration and cell debris near the oviduct (arrows). H. Higher magnification view of region near oviduct in G. Bars = 100 microns, except in C (50 microns).

cell death. This could be due to physiological characteristics unique to stage 7 and older chambers. Alternatively, the stage-specificity might be a coincidence reflecting the time required for the apoptotic phenotypes to develop after an earlier, but undetected, induction. To test these possibilities we used mutations in other genes to block oogenesis at different stages. This will result in the accumulation of immature, but temporally aged, chambers, which can then be examined for their sensitivity to *peg* mutations. In the first set of experiments, we used a mutant allele of the *hu-li tai shao* (*hts*) gene that causes egg chamber arrest prior to stage 7 and is associated with a reduced number of nurse cells (Yue and Spradling, 1992). In our control group, hts^1/hts^+ females mutant for peg^1 displayed the typical *peg* mutant phenotype (Fig. 6A). We compared this phenotype to that found in sibling flies homozygous for both peg^1 and hts^1. In contrast, these females displayed a germline phenotype similar to hts^1 mutants (Fig. 6B,C). The egg chambers were arrested at about stage 4, and all displayed large polyploid nuclei. These results indicate that the *hts* mutation blocks oogenesis before germ cells reach a stage when they become sensitive to aberrant *peg* function.

Similar results were obtained with flies doubly mutant for peg^1 and weak alleles of *otu*. The hypomorphic otu^4 allele typically disrupts oogenesis prior to vitellogenesis, while also causing the formation of nurse cells with polytene, rather than polyploid, chromosomes (King et al., 1986). The distinction between the evenly staining polyploid nuclei and their more irregular polytene counterparts is readily apparent in Feulgen-stained samples (Fig. 6D–F). Females mutant for both otu^4 and peg^1 give a phenotype mostly indistinguishable from otu^4 alone, with little evidence of increase nurse cell death (Fig. 6D,E). However, occasional chambers with pycnotic nuclei are observed, indicating that if the *otu* mutant germline sufficiently mature, they become sensitive to the apoptotic effects of the peg^1 allele (Fig. 6F). This conclusion is supported by parallel studies with otu^5, a weaker allele that typically disrupts oogenesis during stages 8–12 (King et al., 1986). Females mutant of otu^5 and peg^1 display extensive nurse cell degeneration at later oogenic stages, similar to that seen with peg^1 alone (Fig. 6G,H). Taken together, these observations indicate that the peg^1-induced germline death requires the egg chamber to reach a certain level of maturation, approximately correlating with the onset of vitellogenesis.

Somatic follicle cells are not significantly affected by peg *mutations*

The *peg* mutations have profound effects on the development and viability of the female germline, but do not cause obvious morphological defects in the surrounding follicle cells. To better determine whether the somatically derived follicle layer was affected during the initial period of germline aberrations, we examined two functional characteristics, which should be sensitive to any gross physiological abnormalities in the follicle cells.

We first tested the expression of a *Yp2-lacZ* reporter gene in which ß-galactosidase is expressed from the *Yp2* promoter (Bownes et al., 1996). This construct is normally active in follicle cells surrounding the oocyte during stages 8–10, indicating the requirement for stage-specific regulation of promoter activity (Fig. 7A). In peg^1 mutant chambers, the *Yp2-lacZ* reporter displayed a normal expression pattern and time course (Fig. 7B). This was true even in chambers displaying significant nurse cell abnormalities (Fig. 7C), suggesting normal gene regulation in the follicle cells at this time. We also examined a phenomenon known as patency, which describes the pulling away of follicle cells from each other during stages 8–10 (Raikhel and Dhadialla, 1992). This increase in the interfollicular spaces facilitates the passage of yolk spheres from the hemolymph to the oocyte, and can be demonstrated by the trapping of the colloidal dye Trypan Blue within the expanded spaces (Giorgi and Postlethwait, 1985; Mahowald, 1972). We found that wild-type staining was observed in peg^1 mutant egg chambers, consistent with the occurrence of patency and, therefore, normal follicle layer development (Fig. 7D). These experiments suggest that the follicle cells behave normally even during the period when the nurse cells begin their *peg*-induced degeneration.

peg *function is required in the soma, not the germline*

To genetically test whether *peg* expression is required in the germline for normal oogenesis, we performed germline clonal analysis using the dominant female sterile technique (Perrimon and Gans, 1983). Females were made heterozygous for peg^1 and a cell autonomous dominant female-sterile mutation, ovo^{D1}, a genotype that normally results in arrested oogenesis. These females were gamma-irradiated as larvae to induce mitotic crossing over in the germline. Recombination between ovo^{D1} makes possible a mitotic

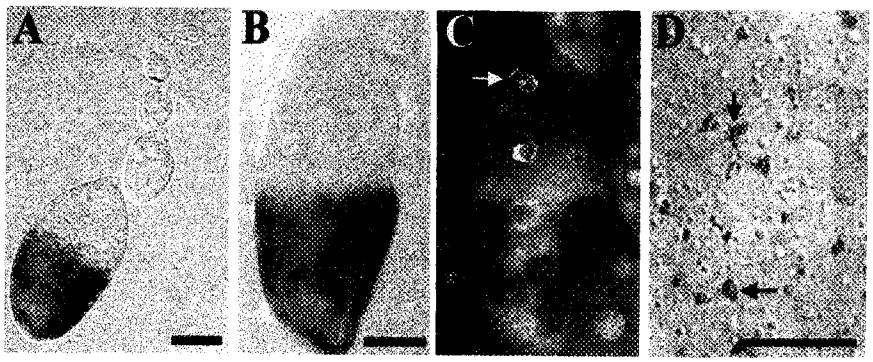

Fig. 7. Follicle cells appear unaffected by *peg* mutations. Ovaries are stained for ß-galactosidase activity and with DAPI. A. The *Yp2-lacZ* reporter expresses ß-galactosidase in follicle cells surrounding the oocyte during stages 8–10 in wild-type chambers. B. The same pattern is observed in *peg* mutant ovaries (w^{1118} peg^1/w^{1118} peg^1; *yp2-lacZ*/+). C. Same preparation as B but with ultraviolet fluorescence to visualize DAPI labeling. Arrow points to aberrant nurse cell nuclei. D. High magnification view of follicle layer surrounding the oocyte of a w^{1118} peg^1/w^{1118} peg^1 stage 9 chamber stained with Trypan blue. Incorporation of the dye in spaces between the follicle cells is an indication of patency (arrows). Bars in A–C = 100 microns. Bar in D = 10 microns.

Table 2. Clonal analysis of the germline

Genotype of irradiated females	# of viable irradiated females	# fertile females	Induction frequency
ovo^{D1} peg^+ *v*/sn^3 ovo^+ peg^1	1240	32*	2.6
fs(1)$Yb^+$$ovo^{D1}$ *v*/*y fs(1)*Yb^1 ovo^+ *cv v f*	2555	82*	3.2

*These were fertile because of the induction of germline clones which lack the dominant female-sterile allele ovo^{D1}. The genotype of the germline clones were shown to be either sn^3 ovo^+ peg^1/sn^3 ovo^+ peg^1 or *y fs(1)*Yb^1 ovo^+ *cv v f*//*y fs(1)*Yb^1 ovo^+ *cv v f*, as confirmed by progeny testing.

segregation pattern that produces a cell homozygous for ovo^+ and the peg^1 mutation. The resultant germline clone will be able to undergo normal oogenesis, provided wild-type *peg* function is not required in the germline (see Materials and Methods).

We found that *peg* activity is not required in the germline for oogenesis. Fertile peg^1, ovo^+ clones were induced in 2.6% of tested females, a recombination frequency approximately equal to that seen in our control cross, and similar to that observed in other studies with this technique (Table 2) (Perrimon and Gans, 1983). Therefore, despite the apparent specificity of the mutant phenotype to the germline, *peg* expression is not necessary in germ cells for their viability or differentiation. These findings suggest that *peg* is required for some soma-germline interaction that is vital to the germline.

Discussion

During oogenesis, a number of interactions between the soma and germline are required for the proper development of a functional egg. These include the regulation of early germline divisions, germline sex determination, and the general organization of the oocyte. Here we report the characterization of a gene required in the soma to maintain germ cell viability, identifying a previously unknown soma-germline interaction.

Mutations in the *peg* gene result in female-sterility associated with the stage-specific degeneration of egg chambers. This process becomes apparent in the nurse cells at oogenic stages 6–7, with the disruption of the microtubule cytoskeleton required for nurse cell-to-oocyte transport. This is rapidly followed by an apoptotic cell death process, involving chromosome fragmentation, nuclear condensation, and ultimately the disintegration of the egg chamber. In contrast to the germline, the somatic components of the ovary and egg chamber appear unaffected prior to the final collapse of the egg.

Despite the specificity of the mutant phenotype to the nurse cells, *peg* expression is not required in the germline for normal oogenesis. This suggests that a somatic process controlled by *peg* is required to maintain the viability of germ cells in vitellogenic egg chambers, an indication of a somatic-germline interaction controlling apoptosis during this period. Interestingly, we observed a similar phenomenon in experiments examining the effects of disruptions in the

follicle layer on the viability and differentiation of the germline (Chao and Nagoshi, 1999). In this study, clonal patches of dying follicle cells were generated, resulting in vitellogenic chambers with large gaps in the follicle layer. The somatic disruption induced apoptotic germline death during stages 7–9, similar to that seen with *peg* mutations. Therefore, it appears that the integrity and proper functioning of the follicle layer are critical for the continued viability of the germline, opening the possibility that this process is influenced by or requires *peg* function.

Our finding that the arrest of germ cell development prior to stage 7 prevented *peg*-induced apoptosis demonstrates that maturation to vitellogenic stages is essential to the cell death process. Similar stage-specificity has also been observed in the germline degeneration caused by mutations in certain early ecdysone-inducible genes (*E74* and *E75*) (Buszczak et al., 1999), leading to the suggestion that an ecdysone-dependent pathway acts as a surveillance mechanism for egg chamber maturation during mid-oogenesis. The existence of a "check point" that monitors the fidelity of oogenesis and eliminates defective eggs by a programmed cell death mechanism has been previously hypothesized (Giorgi and Deri, 1976). Nurse cell apoptosis during vitellogenic stages was also observed in experiments altering the balance of juvenile hormone and 20-hydroecdysone levels in the hemolymph (Soller et al., 1999). The data led to the proposal that both hormones are required to determine whether egg chambers complete normal maturation or become reabsorbed via an induced cell death mechanism (perhaps in response to adverse environmental conditions). Taken together, these observations indicate that germ cells are particularly susceptible to an apoptotic process during stages 7–9 in response to somatic factors.

There is molecular support for this possibility. Foley and Cooley (1998) demonstrated that the expression of apoptotic inhibiting factors (DIAPs) become down regulated in the egg between stages 7 through 9. Although the function of DIAPs have yet to be demonstrated in regulating germline apoptosis, their reduction during the onset of vitellogenesis provides a potential window for cell death induction. In addition, positive regulators of Drosophila apoptosis, i.e., *reaper* and *head-involution-defective*, *dredd*, and *dronc*, are expressed in wildtype chambers beginning at stage 8–9 of oogenesis (Chen et al., 1998; Dorstyn et al., 1999; Foley and Cooley, 1998).

Our findings that *peg* mutations show significant genetic interactions with the $Yp1^{ts1}$ allele suggest the possibility that *peg* may be directly involved in yolk protein synthesis or transport. However, our preliminary results indicate that *peg* mutations do not have obvious effects on the levels of *Yp1* transcript or protein in the ovary, as determined by Northern and Western blot analyses (Fig. 3; data not shown). Furthermore, *Yp1* and *peg* mutations give rise to very different oogenic phenotypes, suggesting that separate processes are affected. For example, $Yp1^{ts1}$ is known to disrupt the integrity of the vitelline membrane and follicle layer, presumably by the formation of aberrant yolk protein granules in the intervening spaces (Butterworth et al., 1992). It would not be surprising if this defect could interfere with a *peg*-dependent soma-germline process, such that the addition of a single copy of the peg^{1} allele leads to sterility.

In summary, there is growing evidence that interactions with the soma are required to maintain the viability of nurse cells, in particular to regulate a germline-specific apoptotic process. We have identified a gene, *peg*, that plays a role in this process. Mutations in *peg* cause the induction of stage-specific nurse cell degeneration without any obvious defects in the soma, yet clonal analysis studies indicate that *peg* expression is not required in the germline for normal oogenesis. Therefore, the *peg* gene appears to be required for a somatic process that acts to maintain the viability of nurse cells, specifically during vitellogenic stages. The mechanism of *peg* function is not known and largely awaits the cloning and molecular characterization of the *peg* gene and product. This is currently in progress and will be the focus of a separate paper.

Acknowledgements

We are grateful to members of the Nagoshi laboratory for helpful discussions. We thank the laboratories of Dr. Ganetzky and Dr. Jan for fly stocks and Dr. M. Bownes for the yolk protein antibody. This work was supported by grants from the National Institutes of Health and the Carver Foundation.

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