

# Regulatory and functional interactions between *ovarian tumor* and *ovo* during *Drosophila* oogenesis

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## Abstract

The *ovo* and *ovarian tumor* genes are required during early and late stages of *Drosophila* oogenesis. The *ovo* product, a zinc-finger transcription factor, can bind to sites and influence the level of expression of the *ovarian tumor* promoter. Our examination of *ovo* null mutant organelles demonstrate that it is required for the differentiation of XX germ cells during larval gonial stages, in addition to its known role in maintaining germ cell numbers. In contrast, *ovarian tumor* is required during pupal and adult stages for the cystocyte divisions that give rise to the egg chamber. Studies on sexually transformed flies indicate that both the *ovo* and *ovarian tumor* null mutant phenotypes are distinctive from and more severe than the germline defects produced when male germ cells develop in female soma. This suggests that *ovo* and *ovarian tumor* have oogenic functions other than their putative role in germline sex determination. We also demonstrate that the regulation of *ovarian tumor* by *ovo* is stage-specific, as *ovarian tumor* promoter activity does not require *ovo* during larval stages but becomes *ovo*-dependent in the adult ovary. This coincides with when the *ovarian tumor* promoter becomes responsive to sex-specific signals from the soma suggesting a convergence of somatic and germline regulatory pathways on *ovarian tumor* during oogenesis. © 1999 Elsevier Science Ireland Ltd. All rights reserved.

**Keywords:** Oogenesis; Germline; *Ovarian tumor*; *Ovo*

## 1. Introduction

In *Drosophila*, the *ovo/shavenbaby* (*ovo/svb*) locus is the site of two functions that are derived by differential RNA processing (Garfinkel et al., 1994; Mèvel-Ninio et al., 1995). One splice form gives rise to the *ovo* product, which is required for female germline development. The alternative *svb* product is required for certain extracellular projections, including some bristles, produced by epidermal cells during embryogenesis. Recently, structural homologs of *ovo* have been identified in mice and humans (Chidambaram et al., 1997; Dai et al., 1998). Knock-outs of the mouse homolog, *movo-1*, displayed aberrations in hair morphology, spermatogenesis, and kidney development. The observations that epidermal appendages and germ cells in both mouse and *Drosophila* require *ovo/svb* products, suggest an evolutionarily conserved genetic pathway extending from insects to mammals (Dai et al., 1998).

Null alleles of *ovo* result in complete female sterility affecting early stages of germline development (Oliver et

al., 1987). During normal oogenesis the larval oogonia differentiate into germline stem cells, which in turn undergo asymmetric divisions to produce daughter stem cells and cystoblasts (King, 1970; reviewed in Spradling, 1993). Each cystoblast undergoes a series of four mitotic divisions to produce a 16 cystocyte syncytium connected by intercellular bridges called ring canals. Upon completion of the cystocyte divisions, somatically derived follicle cells surround the cyst to form the egg chamber. One of the 16 germ cells becomes the oocyte while the remainder differentiate into nurse cells. The maturation of the egg chamber is subdivided into 14 stages based on morphological criteria, with vitellogenesis beginning at stage 8 (King, 1970). The *ovo* null mutant phenotype is associated with a significant reduction in XX germ cell numbers, first consistently observed in larval gonads (Rodesch et al., 1995; Staab and Steinmann-Zwicky, 1995). This results in adult ovaries in which most ovarioles are agametic (Oliver et al., 1987). *ovo* function is also required for later egg maturation, as hypomorphic alleles can block both previtellogenic and vitellogenic development (Oliver et al., 1987).

The OVO protein contains zinc-finger motifs characteristic of certain transcription factors (Mèvel-Ninio et al.,

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1991), and can bind DNA at specific sites in its own promoter and that of the *ovarian tumor (otu)* gene (Lü et al., 1998). Consistent with a positive regulatory function, increases in the levels of *ovo* can increase the in vivo activity of the *otu* promoter (Hager and Cline, 1997; Lü et al., 1998). Like *ovo*, the *otu* gene is required for oogenesis with loss-of-function mutations causing complete female sterility. In *otu* null mutant ovaries most ovarioles are devoid of egg chambers although some ‘ovarian tumors’ (chambers filled with hundreds of immature germ cells) are produced (King, 1969; King and Riley, 1982). Severe hypomorphic alleles increase the frequency of tumor formation, while weaker alleles block during vitellogenic stages (the ‘differentiated’ class, King and Riley, 1982; Storto and King, 1988).

Both the *ovo* and *otu* genes have been implicated in the regulation of sex determination of the germline (Oliver et al., 1987; Oliver et al., 1990; Steinmann-Zwicky, 1992; Oliver et al., 1993; Pauli et al., 1993; Oliver et al., 1994; Wei et al., 1994). This is largely based on the examination of the tumorous germ cells produced by *ovo* and *otu* allele combinations that display some morphological similarities to early spermatocytes and express certain male-specific products. However, this putative sexual transformation by *otu* is, at best, incomplete as *otu*-derived ovarian tumors still express genes specific to (and required for) oogenesis and can produce ring canals with at least some female characteristics (Bae et al., 1994; Rodesch et al., 1997).

Complicating our understanding of oogenesis is the finding that the soma influences the proliferation and differentiation of the germline in a sex-specific manner. When XX germ cells are transplanted into a male soma, they appear to undergo early stages of spermatogenesis (Schüpbach, 1982; Steinmann-Zwicky et al., 1989). Similarly, when XX germ cells develop in an XX soma genetically transformed to a male differentiated state, they can also undergo male-like differentiation (Seidel, 1963; Nöthiger et al., 1989; Steinmann-Zwicky, 1994a). Evidently, the sexual identity of the soma influences oogenesis, perhaps by affecting germline sex determination.

Recently, molecular markers have become available that allow more definitive determination of the differentiated state of germ cells. For example, germline stem cells and cystoblasts can be distinguished from larval oogonial stages by the presence of spectrosomes, an organelle that contains spectrin and f-actin (Lin et al., 1994). As the cystoblast divides, the spectrosome elongates and branches to form a fusome that extends through the ring canals to interconnect the cystocytes. In females, this transition is noted by the disappearance of spectrosomal f-actin during the early cystoblast divisions (Rodesch et al., 1997). In contrast, even mature male fusomes retain detectable levels of f-actin (Hime et al., 1996). Shortly after the completion of the cystocyte divisions, oogonic, but not spermatogenic, ring canals incorporate f-actin and a product of the *hu-li tai shao (hts)* gene (HTS-RC; Lin et al., 1994; Robinson et al., 1994). Therefore, by the appropriate examination of

spectrosomes, fusomes, and ring canals, an approximation of the differentiated and sexual states of mutant germ cells can be determined.

In this manuscript, we identify and describe specific defects in the differentiation of fusomes and ring canals that occur when germ cells of male identity develop in female soma. This indicates that sex-specific somatic interactions are required for the early differentiation of XY germ cells during the cystocyte divisions. The germline aberrations associated with sexually transformed animals were compared with those produced by *ovo* and *otu* null mutations. The results indicate that while *ovo* and *otu* are required for early female germline differentiation, their absence does not induce spermatogenic differentiation in XX germ cells. This has important implications for current models of germline sex determination with respect to *ovo* and *otu* function. In the course of these experiments, we present the first detailed morphological examination of *ovo* null germ cells. The results demonstrate that *ovo* is required for oogonial differentiation in addition to its known role in maintaining XX germ cell numbers. The data provide cytological support for the notion that *ovo* acts prior to *otu* in the differentiation of the female germline. Finally, we examine the pattern of regulatory interactions between *otu* and *ovo*, showing that zygotic *ovo* expression is required for *otu* promoter activity specifically in the adult ovary. The relevance of these findings on our understanding of *ovo* function are discussed.

## 2. Results

### 2.1. XY pseudofemales produce germ cells with aberrant fusomes and ring canals

We recently demonstrated that XX germ cells developing in a male soma produce spermatogenic fusomes (Hinson and Nagoshi, 1999). This indicates at least partial sexual transformation of the XX germline due to sex-specific somatic interactions. In this study, we tested the reciprocal condition, whether the presence of female soma could alter the sexual differentiation of XY germ cells. XY flies were induced to undergo female somatic differentiation by the use of *hs-tra*, a transgenic construct in which the somatic sex determination gene *transformer (tra)* is induced by the *hsp-70* promoter (McKeown et al., 1988). *tra* has no known function in the germline (Schüpbach, 1982), so it is unlikely that *hs-tra* expression directly affects the differentiation of germ cells. XY; *hs-tra*/+ pseudofemales are somatically indistinguishable from XX females, and can support the maturation of transplanted XX germ cells (McKeown et al., 1988; Steinmann-Zwicky, 1994b). In contrast, the endogenous pseudofemale XY germline remains immature, producing tumorous egg chambers that superficially resemble those found in certain *otu* mutants. Surprisingly little is known about the differentiated state of the pseudofemale

germ cells, such as (1) when development is aborted, (2) how sexually dimorphic structures are affected, or (3) their sexual identity.

To define the extent of gametogenic differentiation, the composition of early germline-specific structures were determined by immunohistochemical analyses. All *XY* pseudofemale egg chambers examined contained germ cell clusters with spectrosomes or fusomes ( $n = 10$ , Fig. 1A). The most mature germ cells were interconnected by multi-branched fusomes (polyfusomes) containing f-actin, a phenotype suggestive of spermatogenic differentiation (Fig. 1B1–3). More aberrant germ cells were also found in small (2–3 cell) clusters connected by short, poorly branched fusomes (Fig. 1C1). The retention of f-actin in this subset was variable, indicating an early defect in the spectrosome-to-fusome transition that occurs at the first cystocyte division (Fig. 1C2).

Aberrations were also found in the composition of the ring canals. During normal oogenesis, HTS-RC becomes localized in the rings prior to f-actin and concomitantly with the disappearance of the fusome (Yue and Spradling, 1992). In *XY* pseudofemales, we found that 40% (4/10) of the tumorous chambers examined by phalloidin displayed germ cell clusters with ring canals containing f-actin, but in no case ( $n = 50$  tumorous chambers) did we find HTS-RC deposition (Fig. 1D2). Furthermore, f-actin incorporation in the ring canals occurred while the fusome was still present, a situation not seen in wildtype oogenesis (Fig. 1C1,D1).

These results define a complex and variable set of germ-

line characteristics. The *XY* germ cells typically initiate gametogenic differentiation (to produce spectrosomes and fusomes), but were arrested shortly thereafter, before the differentiation of more mature (post-germarial) stages. A subset of these cells appear to have initiated spermatogenic development, containing male-like polyfusomes consistent with their *XY* genotype. The remainder were more abnormal, with short fusomes and unusual ring canals. We conclude that the presence of female soma disrupts *XY* gametogenesis during the cystocyte divisions, but does not appear capable of inducing oogenic differentiation.

## 2.2. *ovo* is required for differentiation from oogonial to oogenic stages

Null *ovo* mutants show reduced numbers of *XX* germ cells beginning in larval gonads, suggesting either reduced viability or proliferation. Surprisingly little is known about how the absence of *ovo* affects female germ cell differentiation. This is of particular interest given the proposal that mutations in *ovo*, a putative germline sex determination gene, cause the male transformation of *XX* germ cells (Oliver et al., 1990; Oliver et al., 1994). In this case, we might expect *ovo* null mutants to display spermatogenic characteristics similar to that seen in *XY* pseudofemales. Although *ovo* null mutant *XX* germ cells are mostly absent in adult ovaries some can perdure to the adult stage and even form egg chambers, providing the opportunity for their morphological examination (Oliver et al., 1994; Rodesch et al., 1995).

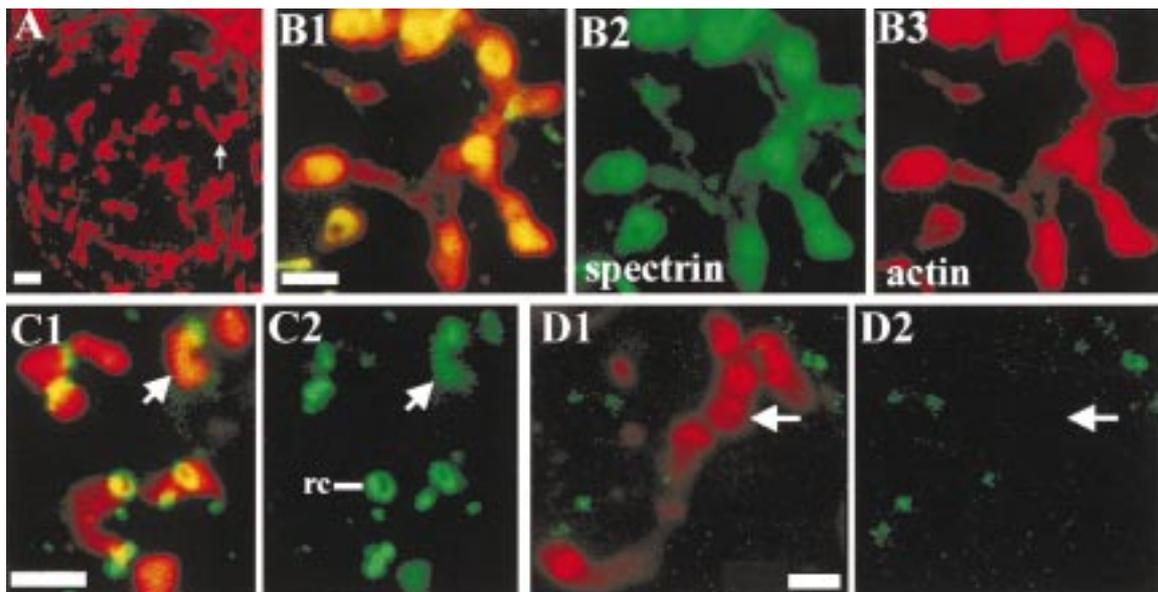


Fig. 1. Germ cell phenotypes of *XY* pseudofemales. The genotype is  $+/B^SY; hs-tra Df(3L)st^{17}, Ki roe pP/+$ . (A) Tumorous egg chamber labeled with anti-spectrin. Several multi-branched fusomes (arrow) are labeled. (B) Fusome from an *XY* pseudofemale tumorous egg chamber labeled with anti-spectrin (Texas red) and phalloidin (Oregon green). (B1) Simultaneous view of both channels. (B2) The Oregon green channel showing presence of spectrin in the fusome. (B3) The Texas red channel showing the presence of f-actin in the fusome. (C1) Pseudofemale fusomes (arrow) and ring canals labeled with anti-spectrin (Texas red) and phalloidin (Oregon green). (C2) Oregon green channel alone showing f-actin in ring canals (rc) and some fusomes (arrow). (D1) Pseudofemale fusomes and ring canals (arrow) labeled with anti-HTS-RC (Oregon green) and phalloidin (Texas red). (D2) Oregon green channel alone showing no HTS-RC in ring canal (arrow). All images from confocal microscopy. Size bars equal 5  $\mu$ M.

In one study, germ cells mutant for the null *ovo*<sup>D1rv23</sup> allele were examined using antibodies specific for spectrin and VASA, a germline-specific protein (Hay et al., 1988). No egg chambers were found, but 20% (5/25) of the ovary lobes contained one or more clusters of VASA-positive germ cells. None ( $n = 12$  VASA-positive clusters) contained either spectrosomes or fusomes although spectrin was present along the cell periphery (Fig. 2A1–2). Similar results were obtained in a separate, larger experiment using *otu-lacZ* as the germline marker. In this construct, the bacterial *lacZ* gene is driven by the germline-specific *otu* promoter (Rodesch et al., 1995). Out of 36 *ovo*<sup>D1rv23</sup> mutant lobes examined, 81% (29/36) had no germ cells, while the remaining seven gonads contained a total of 30 germ cell clusters. The great majority of the clusters (77%, 23/30) contained germ cells with no spectrosomes or fusomes. However, in three ovaries (8%, 3/36) we found a total of seven small germ cell clusters with spectrosomes or fusomes, indicative of gametogenic differentiation (Fig. 2B,C). In these exceptional cases, the fusomes observed were small and poorly branched, indicating aberrant and aborted differentiation early in gametogenesis (Fig. 2C). We conclude that in the absence of *ovo*, the great majority of germ cells abort during larval oögonial stages, affecting both the number of germ cells perduring to the adult stage and their ability to undergo gametogenic differentiation. Therefore, *ovo* null mutant XX germ cells are arrested at an earlier stage than the XY pseudofemale germline, but show no evidence of spermatogenic differentiation.

### 2.3. *otu* mutant fusomes and ring canals differ from those produced in XY pseudofemales

The pseudofemale germline phenotype also differ from that observed in *otu* mutant germ cells. The *otu* null allele, *otu*<sup>PΔ1</sup>, is a deletion of the *otu* coding region and forms either small tumorous egg chambers or chamberless (quiescent) ovarioles (Geyer et al., 1993; Sass et al., 1993). Although the latter often appears agametic, the use of germline molecular markers revealed that virtually all (92/93) *otu* null quiescent ovarioles examined contained germ cell clusters in their germarial regions. Therefore, the quiescent phenotype had been misinterpreted, as *otu* null XX germ cells typically survive and proliferate in adult ovaries.

In each case, the mutant cells had initiated gametogenic differentiation, producing either spectrosomes or short, linear fusomes (Fig. 2D). In our initial description of the *otu* null phenotype (Rodesch et al., 1997), it was noted that the mutant fusomes were, at best, limited to 2-cell clusters and always retained detectable levels of f-actin. Similar results were obtained in our current study, except that the f-actin levels appeared more variable. Phalloidin staining revealed *otu*<sup>PΔ1</sup> mutant fusomes with substantial amounts of f-actin (Fig. 2E1–2), in the same chamber with those with more residual levels (Fig. 2F1–2). It seems likely that the level of f-actin retained in the fusome reflects when the spectrosome-to-fusome transition was blocked by the absence of *otu*. This *otu* null phenotype is similar to the most aberrant class of *otu*<sup>+</sup> XY pseudofemale germ cells,

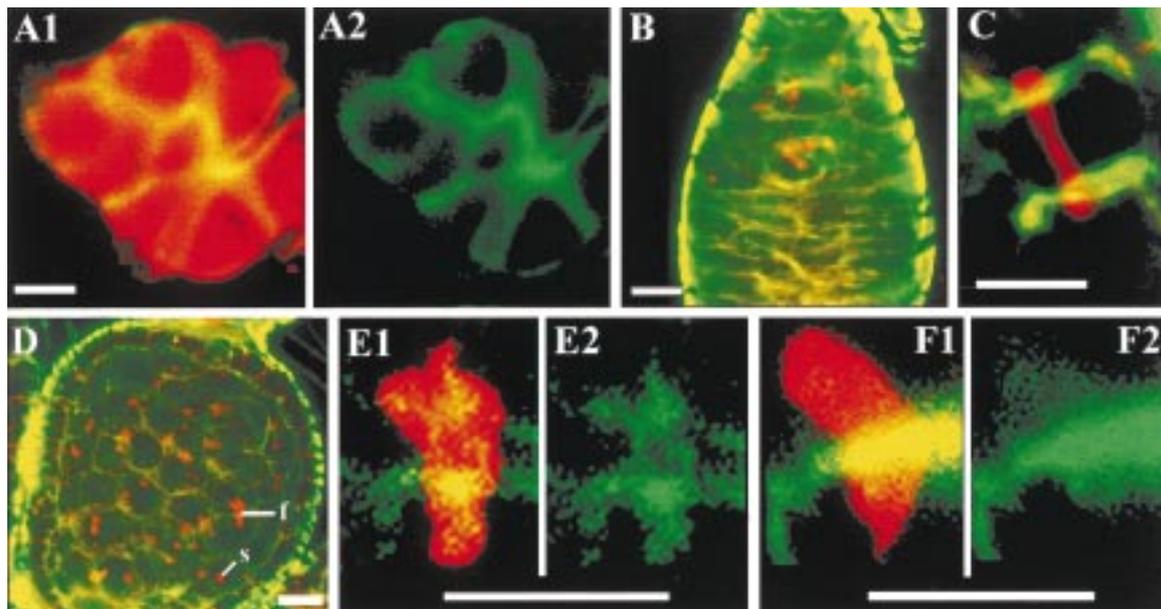


Fig. 2. Germ cell phenotypes of *ovo* and *otu* null mutant germ cells. (A1)  $y w ovo^{D1rv23}/y w ovo^{D1rv23}$  mutant germline labeled with anti-VASA (red) and polyclonal anti-spectrin (green). (A2) Oregon green channel alone showing spectrin labeling only in the cell periphery. There is no evidence of fusomes or spectrosomes. (B,C)  $y w ovo^{D1rv23}/y w ovo^{D1rv23}$  mutant germ cells labeled with anti-spectrin (Texas red) and phalloidin (Oregon green). (B) Germarium with fusomes (arrow). (C) High magnification view of short linear mutant fusome (in red). (D–F) Germ cells from *otu*<sup>PΔ1</sup> *v f/otu*<sup>PΔ1</sup> *v f* tumorous chamber labeled with anti-spectrin (Texas red) and phalloidin (Oregon green). (D) Mutant germ cells contain spectrosomes (s) or short, linear fusomes (f). (E1) High magnification view of *otu*<sup>PΔ1</sup> mutant fusome containing f-actin. (E2) Oregon green channel alone of E1. (F1) High magnification view of *otu*<sup>PΔ1</sup> mutant fusome with reduced levels of f-actin. (F2) Oregon green channel alone of F1. All images from confocal microscopy. Size bars equal 5  $\mu$ M.

which also arrest during the early cystocyte divisions (Fig. 1C). However, unlike in pseudofemales, multi-branched fusomes were never detected nor were the truncated fusomes associated with ring canals containing f-actin (Rodesch et al., 1997; data not shown). From these comparisons, we conclude that the absence of *otu* does not cause XX germ cells to initiate spermatogenic differentiation or become unviable, as previously suggested (Pauli et al., 1993; Mahowald and Wei, 1994).

#### 2.4. *ovo* is required for *otu* promoter activity in adult XX germ cells

Our morphological comparison of *ovo* and *otu* null phenotypes indicate that *ovo* is first required prior to *otu* in female germline development. This timing is consistent with suggestions that *ovo* regulates *otu* promoter activity, as indicated by the finding that dominant, antimorphic *ovo* alleles can reduce *otu* expression (Lü et al., 1998). However,

this observation contradicts our earlier findings that the *otu* promoter is active in the absence of *ovo* function, as *otu-lacZ* is expressed in *ovo* null mutant germ cells (Rodesch et al., 1995). We performed a series of experiments to clarify this regulatory interaction between *ovo* and *otu*.

We first confirmed that *otu* can be expressed in the absence of *ovo*, using a different germline marker. The *pOtu-HA* construct has *otu* tagged with the HA-epitope and expressed from the *otu* promoter. One copy of *pOtu-HA* is capable of rescuing *otu* null alleles to fertility, indicating that it is expressed at all necessary oogenic periods, and displays the expected pattern of OTU protein distribution in the wildtype germline (data not shown). In *ovo<sup>D1rv23</sup>* null mutants, the majority of germ cells expressed substantial levels of the OTU-HA protein, confirming the *otu-lacZ* studies (Fig. 4A).

A different result was observed with hypomorphic *ovo* allele combinations that abort oogenesis during later, post-germarial stages. In these cases the *otu-lacZ* transgene had

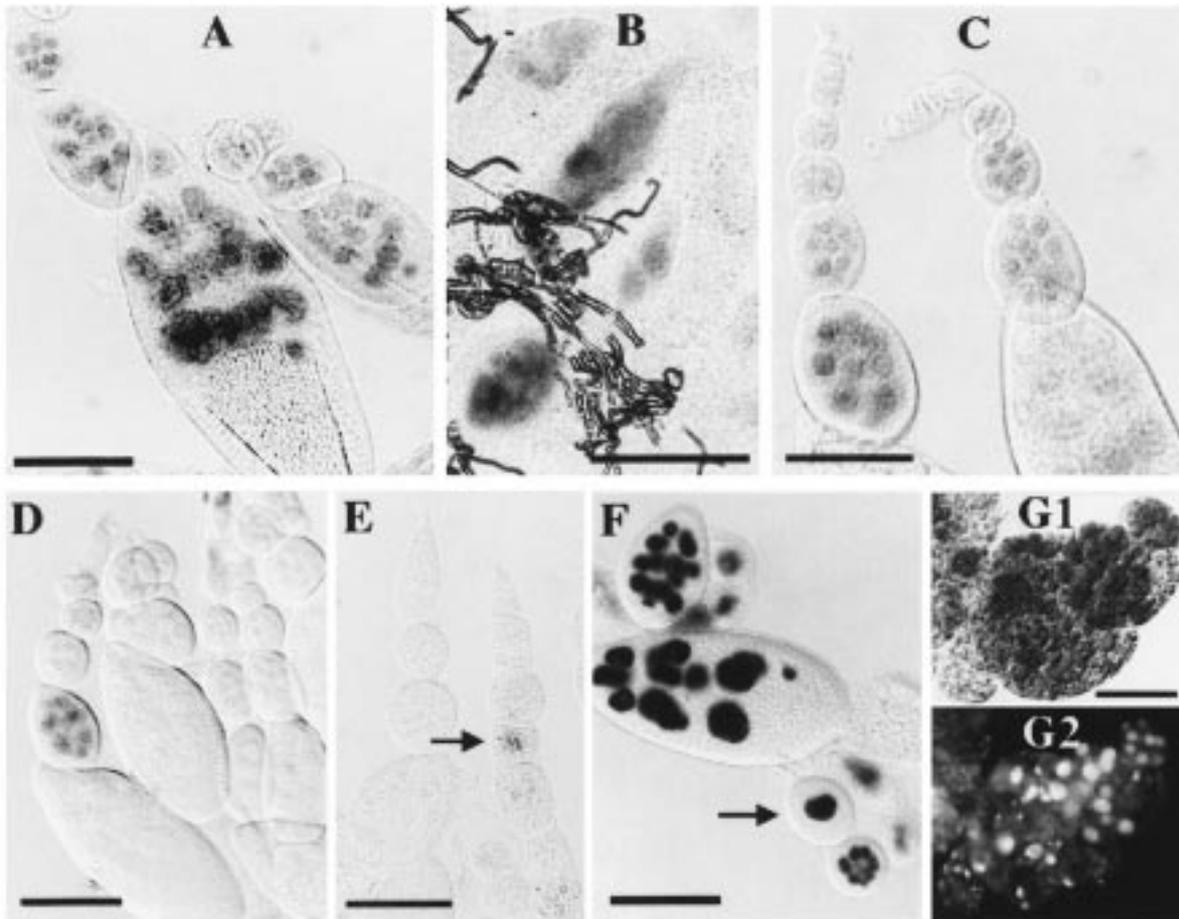


Fig. 3.  $\beta$ -Galactosidase expression in wildtype and mutant germ cells.  $\beta$ -Galactosidase-expressing cells stain dark. All strains carry two copies of *otu-lacZ* unless otherwise noted. (A) Wildtype egg chambers show continuous *otu-lacZ* expression. (B) *y w ovo<sup>D1rv23</sup>/y w ovo<sup>D1rv23</sup>* mutant ovary contain clusters of  $\beta$ -galactosidase-expressing cells. (C) *y ovo<sup>M1</sup>/y ovo<sup>7e</sup>* mutant ovary with low levels of  $\beta$ -galactosidase. (D) *y ovo<sup>M1</sup>/y w ovo<sup>D1rv23</sup>* mutant ovary with one chamber expressing  $\beta$ -galactosidase. (E) *y ovo<sup>7e</sup>/y w ovo<sup>D1rv23</sup>* mutant ovary showing low levels of  $\beta$ -galactosidase-expression in one chamber. (F) *y ovo<sup>M1</sup>/y w ovo<sup>D1rv23</sup>* mutant ovary carrying one copy of *Bc69* (*vasa*-specific enhancer trap line). Arrow points to chamber with single *Bc69*-expressing nurse cell. (G) Disorganized *y cv sov<sup>2</sup>/y cv sov<sup>2</sup>* mutant ovary with *otu-lacZ*. The preparation was stained for  $\beta$ -galactosidase expression and with DAPI. (G1) All germ cells show  $\beta$ -galactosidase-expression despite fusion of ovarioles and egg chambers. (G2) DAPI fluorescence shows presence of large nurse-like nuclei. Size bars equal 100  $\mu$ M.

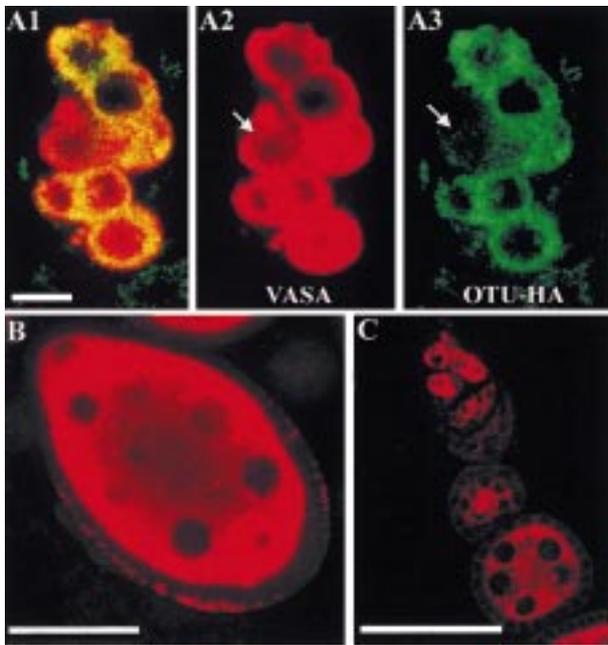


Fig. 4. *otu* promoter expression in *ovo* mutant germ cells. (A) *y w ovo*<sup>D1rv23</sup>/*y w ovo*<sup>D1rv23</sup> mutant germline cluster carrying one copy of *pOtu-HA* labeled with anti-VASA (Oregon green) and anti-HA (Texas red). (A1) Oregon green and Texas red channels. (A2) Texas red channel (VASA) alone. (A3) Oregon green channel (Otu-HA) alone. All images from confocal microscopy. (B,C) *y ovo*<sup>M1</sup>/*y w ovo*<sup>D1rv23</sup> mutant egg chambers carrying one copy of *pOtu-HA* and labeled with anti-HA (Texas red). All images from confocal microscopy. Size bars equal 10  $\mu$ M.

greatly reduced levels of expression, even in relatively mature germ cells that had differentiated into nurse cells and oocytes. Combinations of three *ovo* alleles were examined: *ovo*<sup>M1</sup>, which allows stage 10 and later differentiation, *ovo*<sup>7E</sup> which aborts oogenesis beginning at stage 6, and the null *ovo*<sup>D1rv23</sup> allele. We found that the level of *otu-lacZ* expression was dependent on the severity of the allele combination, with detectable  $\beta$ -galactosidase activity found in *ovo*<sup>M1</sup>/*ovo*<sup>7E</sup> mutants and reduced or undetectable levels present in *ovo*<sup>M1</sup>/*ovo*<sup>D1rv23</sup> and *ovo*<sup>7E</sup>/*ovo*<sup>D1rv23</sup> flies (Fig. 3C,D,E). Interestingly, in the *ovo*<sup>7E</sup>/*ovo*<sup>D1rv23</sup> genotype (where egg chambers were arrested in pre-vitellogenic stages and typically showed no *otu-lacZ* activity) *otu-lacZ* expression was frequently observed in stage 3–4 egg chambers, suggesting a transient period when the *otu* promoter may be less dependent on *ovo* function (arrow, Fig. 3E).

Two approaches were used to examine the specificity of this interaction between *ovo* function and *otu* transcription. In the first case, we showed that *Bc69*, an enhancer-trap line in which *lacZ* is under the control of the germline-specific *vasa* promoter (Laski, personal communication; Rodesch et al., 1995), remained active (Fig. 3F). This was true even in severely aberrant chambers containing only one nurse cell. The second experiment tested the expression of *otu-lacZ* in a different mutant with deformed ovaries. Mutations in the *small ovaries* (*sov*) gene can severely disrupt ovarian and egg chamber development, producing disorganized

mixtures of germline and somatic cells (Wayne et al., 1995). Despite these abnormalities, we found extensive expression of *otu-lacZ* in *sov* mutant nurse cells (Fig. 3G). Therefore, it seems unlikely that the block in *otu* promoter expression by hypomorphic *ovo* alleles is an indirect consequence of abnormal oogenic development. Instead, we believe that upon the development of the adult ovary, there is a significant change in the regulation of *otu* transcription such that it now becomes substantially dependent on positive regulation by *ovo*.

These findings seem at odds with the observation that in the absence of *otu*, female germ cells are arrested during germarial stages. If the hypomorphic *ovo* alleles block the great majority of *otu* gene activity in adult gonads, how can the germ cells differentiate to vitellogenic stages? This was explained by examination of *pOtu-HA* in *ovo*<sup>M1</sup>/*ovo*<sup>D1rv23</sup> mutant ovaries. Despite the *ovo* mutation, the Otu-HA fusion protein was detected during all oogenic stages (Fig. 4B,C). Therefore, *otu* promoter activity in this mutant is sufficient to allow the accumulation of OTU protein.

#### 2.5. The effect of heterologous expression of *otu* in *ovo* mutants

The requirement for *ovo* for the regulation of *otu* promoter activity in adult germ cells suggests the possibility that some or all of the *ovo* post-oogonial mutant phenotype might be due to the reduction in *otu* levels. We tested this possibility by examining the ability of increased *otu* expression to suppress *ovo* mutant oogenic phenotypes. These studies utilized a transgenic construct, *hs-otu*, in which *otu* expression is controlled by the heterologous *Drosophila hsp70* promoter (Rodesch et al., 1995). One copy of *hs-otu* provides sufficient *otu* function to suppress *otu* null alleles at room temperature, allowing egg chamber maturation to late oogenic stages, but typically does not support normal fertility or fecundity (Rodesch et al., 1995; data not shown).

In two separate studies using the same *ovo* mutant strain, females homozygous for the null *ovo*<sup>D1rv23</sup> allele were examined (Table 1, crosses A,B). In one trial, we found no egg chambers in 56 mutant lobes. In the second experiment, 6% of the ovaries contained at least one chamber. In every case the exceptional chambers were small and displayed no nurse-like cells (containing large polyploid nuclei), indicating arrest during early stages of gametogenic differentiation. Similar results were obtained with a second *ovo* null allele, *ovo*<sup>lzl</sup>, where 3% of ovary lobes had at least one chamber (Table 1, cross C).

The addition of *hs-otu* only modestly improved the *ovo* null phenotype. In one study, *ovo*<sup>D1rv23</sup> mutant females with one copy of *hs-otu* resulted in an increase in the proportion of ovary lobes with at least one egg chamber to 37% (Table 1, cross D). However, the degree of this suppression was limited, as the ovaries contained an average of only 3.7 (128/35) chambers/ovary with 91% of these chambers displaying a tumorous phenotype. The remaining 11 cham-

Table 1  
Gonad phenotypes of *ovo* null mutants<sup>a</sup>

Cross	Genotype	Total gonads		Frequency of gonads with:			Total chambers	Chambers with:		Tumorous and nurse cells <sup>b</sup>
		0 chambers	1–5 chambers	> 5 chambers	Only tumorous cells	Tumorous and nurse cells <sup>b</sup>				
A	<i>ovo</i> <sup>-</sup> / <i>ovo</i> <sup>-</sup>	1.00 (56)	0 (0)	0 (0)	8	1.0 (8/8)	0			
B	<i>ovo</i> <sup>-</sup> / <i>ovo</i> <sup>-</sup>	0.94 (66)	0.06 (4)	0 (0)	4	1.0 (4/4)	0			
C	<i>ovo</i> <sup>-</sup> / <i>ovo</i> <sup>-</sup>	0.97 (69)	0.03 (2)	0 (0)	2	1.0 (2/2)	0			
D	<i>ovo</i> <sup>-</sup> / <i>ovo</i> <sup>-</sup> , <i>hs-otu</i> /CyO	0.63 (60)	0.34 (32)	0.03 (3)	128	0.91 (117/128) <sup>c</sup>	0.09 (11/128) <sup>c</sup>			
E	<i>ovo</i> <sup>-</sup> / <i>ovo</i> <sup>-</sup> , <i>hs-otu</i> /CyO	0.66 (19)	0.34 (10)	0 (0)	24	0.96 (23/24)	0.04 (1/24)			

<sup>a</sup> Cross A, B: *y w ovo*<sup>*Δ*123</sup>/*FMO X y w ovo*<sup>*Δ*123</sup>; cross C: *ovo*<sup>*Δ*123</sup>/*vFM6 X ovo*<sup>*Δ*123</sup>/*vFM6*, cross D,E: *y w ovo*<sup>*Δ*123</sup>/*FMO*, *hs-otu*/CyO *X y w ovo*<sup>*Δ*123</sup>, *hs-otu*/CyO.

<sup>b</sup> Nurse cells were identified by the presence of polyploid nuclei.

<sup>c</sup> Includes one exceptional ovary that contained six chambers with nurse cells.

bers from six ovary lobes contained a mix of nurse-like and tumorous cells. The magnitude of suppression was better quantified in a separate experiment using the same *ovo* mutant line. In this case, we examined 29 ovary lobes from *ovo<sup>D1rv23</sup>/ovo<sup>D1rv23</sup>*; *hs-otu*/+ females consisting of over 300 ovarioles (Table 1, cross E). No more than 6% (18/300) of the mutant ovarioles showed evidence of phenotypic suppression by *hs-otu*.

These results were confirmed in a second set of experiments using a more sensitive assay for identifying germ cells. In this case, mutant ovaries were simultaneously labeled with antibodies for the germline-specific VASA protein and HTS-RC. This allowed the detection of germ cells that were not part of egg chambers and provided a molecular marker for oogenic differentiation. Sibling *ovo<sup>D1rv23</sup>* mutant females were examined that carried either zero or one copy of *hs-otu*. In the first study, the presence of *hs-otu* resulted in an increased frequency of germ cell clusters from 0.2 to 2 clusters/lobe (Table 2A). In addition, there was some improvement in the degree of oogenic differentiation as the frequency of germ cell clusters with HTS-RC-positive ring canals (0.1–0.4) or nurse-like cells (0–0.2) also increased. However, chambers remained small and did not progress beyond stage 4. Similar results were obtained in genetic backgrounds that, based on higher baseline frequencies of germ cell clusters, seemed more permissive for the *ovo* mutation (Table 2B,C). Therefore, the additional *otu* activity provided detectable, though limited, improvement in the *ovo* null phenotype. Higher doses of *hs-otu* (2–3 copies) did not result in additional improvement in either the number of germ cell clusters or the degree of oogenic differentiation (Table 2D1,2).

Modest suppression was also observed during later oogenic stages. We tested three different hypomorphic *ovo* allele combinations that allowed some egg deposition, and compared the number of eggs produced in the presence or absence of *hs-otu*. In four independent experiments we found an increase in the number of eggs produced when

*hs-otu* was present, although in no case was hatching observed (Table 3, crosses A–D). Taken together, these results suggest that *ovo*-independent expression of *otu* can ameliorate some aspects of the *ovo* mutant phenotype. However, this suppression is quite limited, suggesting that *ovo* has other necessary oogenic functions besides regulating *otu* promoter activity.

### 3. Discussion

Our experiments on XY pseudofemales make several contributions toward understanding how the soma and germline interact to regulate gametogenesis. Firstly, we found that the presence of female soma caused XY germ cells to abort development during the cystocyte divisions, the same period at which XX germ cells arrest when developing in male soma (Hinson and Nagoshi, 1999). This demonstrates a common need for sex-specific somatic interactions during the period of fusome and ring canal morphogenesis in both sexes. Secondly, the presence of female soma did not promote oogenic fusome differentiation in XY germ cells. In comparison, male soma was able to induce a subset XX germ cells to produce spermatogenic polyfusomes (Hinson and Nagoshi, 1999). This suggests possible differences in how male and female soma can influence the determination of sexual identity in the germline. Thirdly, the presence of female soma had an unexpected effect on the ring canals of XY germ cells, producing a phenotype we had never observed in other genotypes. A number of pseudofemale ring canals contained f-actin, but not HTS-RC. This is particularly unusual because HTS-RC is normally deposited before f-actin during oogenesis (Yue and Spradling, 1992). Therefore, the sexual incompatibility between the germline and soma seems to have a specific effect on the deposition of f-actin in the maturing ring canal. Interestingly, this correlates with our observations that *otu* is itself regulated by somatic interactions (Hinson and Nagoshi,

Table 2  
Suppression of *ovo* nulls by heterogeneous *otu* expression<sup>a</sup>

	Genotype	Total ovary lobes	Clusters/lobe	Frequency of clusters with HTS-RC	Frequency of clusters with nurse-like cells
A1	<i>ovo<sup>-</sup>/ovo<sup>-</sup></i>	37	0.2 (9/37)	0.1 (1/9)	0 (0/9)
A2	<i>ovo<sup>-</sup>/ovo<sup>-</sup>+1 hs-otu</i>	35	2.0 (70/35)	0.4 (31/70)	0.2 (14/70)
B1	<i>ovo<sup>-</sup>/ovo<sup>-</sup></i>	20	14.7 (293/20)	0.3 (99/293)	0.01 (4/293)
B2	<i>ovo<sup>-</sup>/ovo<sup>-</sup>+1 hs-otu</i>	25	10.2 (254/25)	0.6 (161/254)	0.07 (18/254)
C1	<i>ovo<sup>-</sup>/ovo<sup>-</sup></i>	14	12.5 (175/14)	0.2 (39/175)	0.03 (6/175)
C2	<i>ovo<sup>-</sup>/ovo<sup>-</sup>+1 hs-otu</i>	13	3.6 (47/13)	0.4 (19/47)	0.17 (8/47)
D1	<i>ovo<sup>-</sup>/ovo<sup>-</sup>+2 hs-otu</i>	18	6.0 (108/18)	0.5 (56/108)	0.16 (17/108)
D2	<i>ovo<sup>-</sup>/ovo<sup>-</sup>+3 hs-otu</i>	22	6.3 (139/22)	0.6 (77/139)	0.14 (19/139)

<sup>a</sup> Cross A: *w ovo<sup>D1rv23</sup>/FMO; hs-otu/CyO X y w ovo<sup>D1rv23</sup>/Y*. Cross B: *y w ovo<sup>D1rv23</sup>/FMO; hs-otu/CyO X y w ovo<sup>D1rv23</sup>/Y; KZ503/TM3* (KZ503 is a *kinesin-lacZ* fusion construct that was incidental to these experiments). Cross C: *y w ovo<sup>D1rv23</sup>/FMO; hs-otu/CyO X y w ovo<sup>D1rv23</sup>/y; otu-lacZ*. Cross D: *y w ovo<sup>D1rv23</sup>/FMO; hs-otu/CyO; hs-otu/TM3 Ser X y ovo<sup>D1rv23</sup>/Y; hs-otu/CyO; hs-otu/TM3 Ser*. For D2, all females examined were *y w ovo<sup>D1rv23</sup>/y w ovo<sup>D1rv23</sup>; hs-otu/hs-otu; hs-otu/TM3 Ser*. Homozygotes of the third chromosome *hs-otu* construct were infrequent and therefore not counted in these experiments.

Table 3  
Partial suppression of the late *ovo* mutant phenotype<sup>a</sup>

	Genotype	# Females tested	Eggs/female	% Hatch
A1	<i>ovo</i> <sup>M1</sup> / <i>ovo</i> <sup>D1rv23</sup> ; +/CyO	100	4.7	0
A2	<i>ovo</i> <sup>M1</sup> / <i>ovo</i> <sup>D1rv23</sup> ; +/ <i>hs-otu</i>	100	6.9	0
B1	<i>ovo</i> <sup>M1</sup> / <i>ovo</i> <sup>D1rv23</sup> ; +/+	30	20.9	0
B2	<i>ovo</i> <sup>M1</sup> / <i>ovo</i> <sup>D1rv23</sup> ; +/ <i>hs-otu</i>	20	43.5	0
C1	<i>ovo</i> <sup>M2</sup> / <i>ovo</i> <sup>D1rv23</sup> ; +/+	100	15.2	0
C2	<i>ovo</i> <sup>M2</sup> / <i>ovo</i> <sup>D1rv23</sup> ; +/ <i>hs-otu</i>	40	38.7	0
D1	<i>ovo</i> <sup>8F</sup> / <i>ovo</i> <sup>D1rv23</sup> ; +/+	16	1.8	0
D2	<i>ovo</i> <sup>8F</sup> / <i>ovo</i> <sup>D1rv23</sup> ; +/ <i>hs-otu</i>	12	51.8	0

<sup>a</sup> Each female class was heat shocked for 1 h at 37°C in an air incubator 1 day before mating to an equal number of wildtype males. All crosses performed at 25°C. Cross A: *y w ovo*<sup>D1rv23</sup>/*FM6*; *hs-otu*/CyO *X y ovo*<sup>M2</sup>/*Y, KZ503/TM3*. *Cy*<sup>+</sup> and *Cy ovo* mutant sibling females were examined. We became concerned that the *CyO* chromosome might be modifying the *ovo* phenotype, so we modified crosses B–D in the following manner (note that *ovo*<sup>Z</sup> represents in Cross B: *ovo*<sup>M1</sup> Cross C: *ovo*<sup>M2</sup>, and for Cross D: *ovo*<sup>8F</sup>). *y w ovo*<sup>D1rv23</sup>/*FM6*; *hs-otu*/CyO females were crossed to *y ovo*<sup>Z</sup>/*Y*; +/+. *y w ovo*<sup>D1rv23</sup>/*y ovo*<sup>Z</sup>; *hs-otu*+ daughters were tested for egg lay (Crosses B2, C2, D2). For the B1–D1 controls, sibling females of the genotype *y ovo*<sup>Z</sup>/*FM6*; +/CyO were crossed to *y w ovo*<sup>D1rv23</sup>/*Y*; +/+ males. *y w ovo*<sup>D1rv23</sup>/*y ovo*<sup>Z</sup>; +/+ progeny now no longer carried *CyO* and were tested for fertility and egg lay.

1999), and is required for f-actin deposition in the ring canals (Rodesch et al., 1997).

In addition, the pseudofemale studies identified the phenotype to be expected when male germ cells develop in female soma. This provided an important comparison for our examination of the *ovo* null phenotype and the proposal that *ovo* is required for germline sex determination (Oliver et al., 1987; Oliver et al., 1990; Oliver et al., 1993). The hypothesis predicts that XX germ cells lacking *ovo* function will take on a male identity, and therefore should display some spermatogenic characteristics. In contrast, we found that those *ovo* null germ cells perduring in adult ovaries typically lacked spectrosomes, fusomes, or ring canals. This indicates arrest during the larval oogonial stages, the same period when *ovo* null mutants first show reductions in germ cell numbers (Rodesch et al., 1995; Staab and Steinmann-Zwicky, 1995). Such a developmental block could readily give rise to the adult *ovo* null agametic phenotype. For example, the oogonia are a period of proliferation, so an arrest at this time would markedly reduce germ cell numbers. In addition, the immature mutant germ cells might fail to associate normally with the somatic ovary as it differentiates during late larval and pupal periods. Therefore, the *ovo* null phenotype could be explained by *ovo* being required simply for early oogonial differentiation, without having to invoke a role in germline sex determination. A later arrest is observed in *otu* null mutants, where oogenesis is aborted during the first cystocyte division. Therefore, *otu* is initially required after the period when *ovo* first acts. This temporal ordering is consistent with recent evidence that *ovo* controls *otu* transcription (Lü et al., 1998). Our experiments demonstrate that this regulatory interaction is a complicated one. During larval stages, the *otu* promoter is expressed in both male and female gonads and does not appear to require *ovo* (Rodesch et al., 1995; Hinson and Nagoshi, 1999), although the possibility of regulation by maternally contributed *ovo* product cannot be precluded (Mével-Ninio et al., 1995). It is only in the

adult gonad that *otu* promoter activity becomes dependent on zygotic *ovo* expression. Coincidentally, the adult stage also defines two other changes in *otu* regulation. It is only in adults that the *otu* promoter exhibits female-specific expression and dependence on sex-specific somatic interactions (Fig. 5). In fact, we previously demonstrated that the inhibitory effects of male soma on oogenesis is due primarily to insufficient *otu* expression (Nagoshi et al., 1995; Hinson and Nagoshi, 1999). Therefore, the formation of the adult ovary correlates with a change in *otu* regulation such that it now becomes the target of both *ovo*- and soma-dependent inputs. In this way, *otu* may serve to coordinate the development of germline and somatic components of the egg chamber.

## 4. Materials and methods

### 4.1. Fly strains and mutations

Flies were raised on a standard cornmeal, molasses, yeast, agar media containing propionic acid as a mold inhibitor

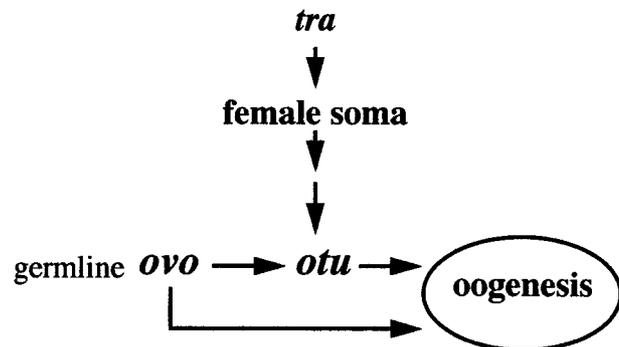


Fig. 5. Proposed pathway for the regulation of *otu* promoter activity. In adult ovaries, the *otu* promoter activity in the germline requires the function of the germline-specific *ovo* gene and sex-specific interactions with the soma. There also appears to be at least one *ovo* oogenic function that is independent of *otu*.

and supplemented with live yeast. Alleles and chromosomes used are described in Lindsley and Zimm (1992). *otu*<sup>PΔ1</sup> is a deletion of the entire *otu* coding region (Geyer et al., 1993; Sass et al., 1993). *ovo*<sup>D1rv23</sup> (also called *ovo*<sup>D1rs1</sup>) is a complete loss of function allele (Oliver et al., 1987), associated with an insertion that probably leads to premature transcription termination (Garfinkel et al., 1992). *ovo*<sup>lzl</sup> is an insertion mutation believed to be a complete null for the germline function (Mèvel-Ninio et al., 1989; Garfinkel et al., 1992). *ovo*<sup>M1</sup>, *ovo*<sup>M2</sup>, *ovo*<sup>7E</sup> and *ovo*<sup>8F</sup> are partial loss of function alleles induced by EMS (Mohler, 1977; Oliver et al., 1987; Salz, 1992). XY pseudofemales were created by the crosses, (a) *otu*<sup>+</sup>, *ovo*<sup>+</sup> XY pseudofemales: +/+ , *hs-tra* *Df(3L)st<sup>i7</sup>*, *Ki roe pP/TM6* females X y w *ovo*<sup>D1rv23</sup>/*B<sup>S</sup>Y* males and collecting +/*B<sup>S</sup>Y*; *hs-tra Df(3L)st<sup>i7</sup> Ki roe pP/* +; (b) *otu* null pseudofemales: *otu*<sup>PΔ1</sup> v *ff/FM6*; *hs-tra Df(3L)st<sup>i7</sup> Ki roe pP/TM6* X y w *ovo*<sup>D1rv23</sup>/*B<sup>S</sup>Y*; (c) *ovo* null pseudofemales: y w *ovo*<sup>D1rv23</sup>/*F<sup>M</sup>6*; *hs-tra Df(3L)st<sup>i7</sup> Ki roe pP/TM6* X y w *ovo*<sup>D1rv23</sup>/*B<sup>S</sup>Y*. The relevant crosses for Fig. 4 included: (i) y w *ovo*<sup>D1rv23</sup>/*F<sup>M</sup>6*; *otu-lacZ/CyO* X y w *ovo*<sup>D1rv23</sup>/*Y*; *otu-lacZ/CyO*, (ii) y *ovo*<sup>M1</sup>/*F<sup>M</sup>6*; *otu-lacZ/CyO* X y *ovo*<sup>7E</sup>/*Y* *otu-lacZ/CyO*, (iii) y w *ovo*<sup>M1</sup>/*F<sup>M</sup>6*; *otu-lacZ/CyO* X y w *ovo*<sup>D1rv23</sup>/*Y* *otu-lacZ/CyO*, (iv) y v *ovo*<sup>M1</sup>/*F<sup>M</sup>6* X y w *ovo*<sup>D1rv23</sup>/*Y*; *Bc69/CyO*, (v) y *cv sov*<sup>2</sup>/*F<sup>M</sup>6*; *otu-lacZ/CyO* X y *cv sov*<sup>2</sup> *otu-lacZ/CyO*. The remaining crosses are described in Table 1, Table 2 and Table 3.

#### 4.2. Examination of *ovo* and *otu* mutant phenotypes

Morphological studies on *ovo* and *otu* null mutants were performed on adult females aged 2–5 days post-eclosion (at room temperature). We found that this time period gave the highest frequency of germ cell clusters. All crosses were cultured at room temperature (20–23°C) or at 25°C unless otherwise noted. Heat shock conditions (exposure to >27°C) were not utilized for experiments with *ovo* null alleles as higher temperatures tended to exacerbate the *ovo* null phenotype (Oliver et al., 1994; data not shown).

#### 4.3. Transgenic constructs

*pOtu-HA* is an epitope-tagged transgene in which the genomic *otu* sequence is fused to three tandem copies of the nine amino acid HA epitope (Boehringer Mannheim). To construct *pOtu-HA*, polymerase chain reaction methodologies were used to amplify the tandemly repeated HA sequence from *pMPY-3XHA* (Schneider et al., 1995). A PCR fragment with three copies of HA and *Mlu*I-compatible *Asc*I ends was created and inserted into a subclone containing the 3' *Xba*I-*Pst*I portion of *otu* in the *w*<sup>+</sup> *Drosophila* transformation vector *Casper-3* (Pirrotta, 1988). The HA repeat was fused in frame to the terminal *otu* codon and upstream of the *otu* polyadenylation signal, forming *pOtu-HA.X-P*. An *Xba*I fragment containing the remaining genomic portion of the *otu* gene, including 640 bp of promoter sequence upstream of the primary transcription start site was inserted into the *Xba*I site of *pOtuHA.X-P*, creating *pOtu-*

*HA*. Germline transformation of *pOtu-HA* was carried out by standard methods (Rubin and Spradling, 1982). The *hs-tra* and *hs-otu* transgenes place *tra* and *otu* under the control of the *Drosophila hsp70* promoter and are marked with *ry*<sup>+</sup> (McKeown et al., 1988; Hinson and Nagoshi, 1999). *hs-tra* is on the third chromosome and can cause female differentiation of XY soma at room temperature. The *hs-otu* construct used is inserted onto an unmarked second chromosome and can suppress the *otu* null phenotype at room temperature. *otu-lacZ* is a *w*<sup>+</sup> transgene in which the *otu* promoter is fused to *lacZ* (Rodesch et al., 1995). *otu-lacZ* strains with an insertion on the 2nd or 3rd chromosomes were used.

#### 4.4. Whole mount immunohistochemistry and fluorescent labeling

Adult gonads were dissected in PBS (130 mM NaCl, 7 mM Na<sub>2</sub>HPO<sub>4</sub>·2H<sub>2</sub>O, 3 mM NaH<sub>2</sub>PO<sub>4</sub>·2H<sub>2</sub>O). The tissues were fixed in a 1:1 solution of fix:heptane (fix: 4% EM grade paraformaldehyde in PBS) for 20 min with gentle agitation. The tissues were washed 3 × in PBT (0.1% Triton X-100, 0.05% Tween 80 in PBS) for 15 min each. Tissue preparations were permeabilized for at least 2 h in PBT and blocked overnight in PBT+1 mg/ml crystalline BSA (Sigma) at 4°C. Preparations were then incubated with primary antibodies (appropriately diluted in PBT/BSA) at 4°C overnight. Secondary antibodies were diluted 1:200 in PBT/BSA and incubated with tissues for at least 3 h at room temperature. Primary monoclonal antibodies included: anti- $\alpha$ -spectrin (3A9; 1:200 dilution; gift from D. Branton and distributed by the University of Iowa Developmental Studies Hybridoma Bank), anti-HTS-RC (HTS 655 4C; 1:100 dilution), and anti- $\beta$ -galactosidase (mouse IgG monoclonal 40-1a from the University of Iowa Developmental Studies Hybridoma Bank: 1:100 dilution). Polyclonal antibodies included anti-VASA (L. and Y. Jan; 1:1000). Secondary antibodies used were Oregon green or Texas red-conjugated anti-mouse or anti-rabbit IgG (1:200; Molecular Probes). Phalloidin staining was achieved by dissolving 2 units of either Texas red or Oregon green-conjugated phalloidin (Molecular Probes) in 200 ml PBT/BSA and incubating for 30 min at room temperature. Phalloidin conjugates were removed by rinsing three times for 15 min in PBT. Labeled preparations were mounted in Vectashield (Vector Laboratories). When necessary, nuclei were labeled by propidium iodide. In this case, tissue preparations were incubated in 125  $\mu$ g/ml RNase (Boehringer Mannheim) in PBT for 1 h before incubation with secondary antibodies. When necessary, nuclei were labeled with the fluorescent dye DAPI by incubating preparations in 0.2 mg/ml DAPI in PBT for 30 min. Tissues were mounted in Vectashield (Vector Laboratories).

#### 4.5. $\beta$ -Galactosidase staining

Gonads were dissected in PBS, then incubated in 50%

fixative: 50% heptane in a covered depression slide with agitation for 3 min. The tissue was rinsed 3× in PBS+0.1% Triton X-100. The tissue was incubated in staining solution overnight at 37°C in the dark. After staining, the preparation was washed 5× for 20 min with PBS. The tissue was mounted in 50% glycerol in PBS. Stock solutions: Solution A, 6.75 g/l NaCl, 6.63 g/l KCl, 0.66 g/l MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.54 g/l MgCl<sub>2</sub>·6H<sub>2</sub>O; 0.33 g/l CaCl<sub>2</sub>·2H<sub>2</sub>O; Solution B, 1.4 g/l Na<sub>2</sub>HPO<sub>4</sub>, 0.1 g/l KH<sub>2</sub>PO<sub>4</sub>, taken to pH 7 with NaOH; and Solution C, the same as Solution A but with 3.7% formaldehyde. Fixative: 9 parts Solution C and 10 parts Solution B. Staining solution: 0.75 ml of a mixture of 9 parts Solution A and 10 parts Solution B, 0.1 ml 50 mM potassium ferricyanate, 0.1 ml 50 mM potassium ferrocyanate, 50 μl 100 μg/μl 5-bromo-4-chloro-3-indoxyl-β-galactopyranoside (X-gal) in N,N'-dimethylformamide, to a total volume of 1.0 ml in water.

#### 4.6. Microscopy and image analysis

Confocal images were obtained on a Nikon Optiphot using a Bio-Rad MRC 1024 confocal laser apparatus. Sections were manipulated using Bio-Rad Lasersharp image analysis software. Other microscopy was performed on an Olympus Vanox AHB3 microscope using an Optronics LX4SOA camera for image capture. Figures were produced by transferring captured images to Adobe Photoshop 4.0. All processing (i.e. changes in contrast, brightness, etc.) were performed simultaneously over the entire image. Therefore, within each plate, the relative signal intensities between different areas are as originally captured.

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