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ovarian tumor expression is dependent on the functions of the somatic sex regulatory genes *transformer-2* and *doublesex*

Shannon Hinson[1], Rod N. Nagoshi*

Department of Biological Sciences, University of Iowa, Iowa City, IA 52242-1234, USA

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Abstract

The *doublesex*-dependent sex regulatory pathway in *Drosophila* controls major aspects of somatic sexual differentiation, but its expression is not required in the *X*/*X* germline. Nevertheless, mutations in *doublesex* and in the genes that directly regulate its expression, *transformer* and *transformer-2*, disrupt early stages of oogenic differentiation to produce gonads containing immature germ cells. This indicates a critical, but uncharacterized, set of soma–germline interactions essential for oogenesis. In this paper, we examined the effects of mutations in *transformer-2* on the expression and function of the germline-specific *ovarian tumor* gene. We demonstrated that in *transformer-2* mutants, there was a marked reduction in the activity of the *ovarian tumor* promoter in the mutant germline. In addition, the phenotypic effects on the arrested germline could be partially suppressed by the simultaneous over-expression of both *ovarian tumor* and a second germline gene, *Sex-lethal*. This differs from *transformer* mutations, in which the over-expression of *ovarian tumor* alone is sufficient for a similar improvement in germline differentiation. In contrast to *transformer-2*, *doublesex* activity was not required for *ovarian tumor* promoter activity and we found indirect evidence that the *doublesex* male-specific function might have a negative regulatory effect. These data indicate that the components of the genetic pathway regulating somatic sexual differentiation have specific and differential effects on germline gene activity.

Keywords: Oogenesis; *transformer-2*; *ovarian tumor*; *transformer*; *Sex-lethal*

1. Introduction

In *Drosophila melanogaster*, a female ratio of X-chromosomes to the set of autosomes (X/A = 2X/2A) initiates a pathway leading to the expression of the active *transformer* (*tra*) product (McKeown et al., 1987). The *tra* function acts together with the product of the constitutive *transformer-2* (*tra2*) gene to regulate two subordinate pathways, one controlled by *doublesex* (*dsx*) and the other by *fruitless* (*fru*; Heinrichs et al., 1998; Nagoshi et al., 1988; Yamamoto et al., 1998). The *dsx* gene produces sex-specific products required in both males and females for the development of a subset of sexually dimorphic structures, including the somatic gonad (Baker and Ridge, 1980). The *tra*, *tra2*, and *dsx* genes act cell autonomously in the soma, and both pole cell transplantation and clonal analysis experiments demonstrate that none needs to be expressed in the germline for normal oogenesis (Marsh and Wieschaus, 1978; Schüpbach, 1982).

Despite its tissue-specificity, the somatic sex regulatory pathway plays a critical role in the differentiation of the *X*/*X* germline. Loss-of-function mutations in *tra* or *tra2* cause a male transformation of *X*/*X* flies to produce 'pseudomales' that are somatically similar to *X*/*Y* males (Baker and Ridge, 1980; Fujihara et al., 1978; Sturtevant, 1945; Watanabe, 1975). However, these sexually transformed animals are sterile, with small, uncoiled 'pseudotestes' containing undifferentiated germ cells (Nöthiger et al., 1989; Seidel, 1963). Mutant allele combinations of *dsx* can also generate *X*/*X* pseudomales or (in the case of loss-of-function mutations) *X*/*Y* or *X*/*X* intersexes in which an intermediate sexual phenotype occurs (Baker and Ridge, 1980; Hildreth, 1965). In both cases gametogenesis is aborted early and the gonads appear small and degenerate (Hildreth, 1965; Nöthiger et al., 1980). Morphological examination of *X*/*X* pseudomale gonads identified a subset of germ cells with spermatogenic characteristics, suggesting that male-specific somatic factors can alter the sexual identity indicated by the germ cell's own X:A ratio (Hinson and Nagoshi, 1999;

* Corresponding author. Address: USDA-ARS CMAVE, P.O. Box 14565, 1600/1700 SW 23rd Drive, Gainesville, FL 32604, USA. Tel.: +1-352-374-5779; fax: +1-352-374-5804.

E-mail address: rodney-nagoshi@uiowa.edu (R.N. Nagoshi).

[1] Address: Mayo Clinic Rochester, Rochester, MN 55905, USA.

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Nöthiger et al., 1989; Steinmann-Zwicky et al., 1989). If correct, it would indicate a more complex sex determination mechanism than found in the soma, with germline sexual identity defined by a combination of cell autonomous and non-autonomous regulatory influences.

Among the germline-specific genes implicated in regulating the early female differentiation of germ cells is *ovarian tumor* (*otu*), which is required during several oogenic stages (King and Riley, 1982; Storto and King, 1988). Germline stem cells normally undergo asymmetric divisions to form daughter stem cells and cystoblasts. These two cell types can be identified by the presence of a spherical, spectrin-rich spectrosome (Lin and Spradling, 1995). The cystoblast undergoes a set of four mitotic divisions to produce 16 cystocytes connected by ring canals through which passes the fusome, a multi-branched derivative of the spectrosome (Lin and Spradling, 1995; Lin et al., 1994). Shortly after the formation of the 16-cystocyte cluster, the fusome disappears and the cyst becomes enveloped by the somatically derived follicle cells to form the egg chamber.

X/X germ cells mutant for null *otu* alleles abort oogenesis at about the first cystoblast division (Rodesch et al., 1997). This produces either a 'quiescent' phenotype where ovarioles are devoid of egg chambers (but still typically contain germ cells in their germarial regions) or small 'ovarian tumors' in which egg chambers are filled with hundreds of poorly differentiated germ cells (Geyer et al., 1993; Rodesch et al., 1995; Storto and King, 1988). Severe hypomorphic *otu* mutations produce larger ovarian tumors associated with arrest during later cystocyte divisions, while weaker alleles allow formation of differentiated egg chambers arrested during late vitellogenic stages (King et al., 1986; King and Riley, 1982; Rodesch et al., 1997).

In this paper, we examine how the somatic sex regulatory pathway influences the regulation and germline-specific functions of the *otu* gene. We previously demonstrated that *tra* somatic function is required to maintain *otu* promoter activity in the germline, thereby linking the somatic and germline regulatory pathways controlling sexual differentiation (Hinson and Nagoshi, 1999). We now extend these observations to show that *tra2* mutations repress *otu* activity in the same way. However, *tra* and *tra2* mutations differ in one important aspect. While germ cells from *tra* mutant pseudomales can be induced to undergo further differentiation by the overexpression of *otu* alone (Hinson and Nagoshi, 1999), *tra2* mutant germ cells are not affected. Instead, they seem to require the simultaneous overexpression of *otu* and *Sxl* to induce further oogenic differentiation.

2. Materials and methods

2.1. Fly strains

Flies were raised on standard cornmeal, molasses, yeast, and agar media containing propionic acid as a mold inhibitor and supplemented with live yeast. Unless otherwise noted, alleles and chromosomes used are as described (Lindsley and Zimm, 1992). The *hs-otu* construct places *otu* function under the control of the *Drosophila hsp70* promoter and was previously described (Nagoshi et al., 1995). The ry^+ transgene is inserted into a third chromosome that is *ry* and *e*. The *pOtu-lacZ* strain contains the w^+ marked transgene inserted into an unmarked third chromosome (Rodesch et al., 1995). The construct consists of *otu* promoter sequences −1027 to +63 fused to the bacterial *lacZ* gene. *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).

$otu^{P\Delta 1}$ is a deletion of the entire *otu* coding region (Geyer et al., 1993; Sass et al., 1993). Females that are homozygous mutant for this allele are completely sterile with a tumorous or quiescent ovary phenotype. The chromosome is marked with *v* and *f*. $otu^{P\Delta 3}$ and $otu^{P\Delta 5}$ are two different deletions in the *otu* promoter that reduce transcription levels to different degrees (Geyer et al., 1993; Sass et al., 1993). otu^2 is a severe allele that produces primarily quiescent ovaries when homozygous (King and Riley, 1982; Storto and King, 1988).

2.2. Construction of X/X pseudomales

X/X flies that carry mutations in *tra* develop as pseudomales. The heterozygous combinations of two loss-of-function alleles, tra^1 and tra^4, were used to increase viability of pseudomales (McKeown et al., 1987). The tra^4 chromosome is marked with kar^2 ry^5 and *red* and the tra^1 chromosome is unmarked. *tra* mutant pseudomales that carried one copy of *hs-otu* were generated by mating *hs-otu/hs-otu*; tra^4/*TM6* females to +/$B^s Y$;; tra^1/*TM6* males.

X/X flies that carry mutations in *tra2* also develop as somatic males. Heterozygous combinations of two loss-of-function alleles ($tra2^1$ and $tra2^B$) were used to increase viability of pseudomales (crosses are described in Table 1). The $tra2^B$ chromosome is marked with *cn* and *bw*. Both $tra2^1$ and $tra2^B$ are loss-of-function alleles (Baker and Ridge, 1980; Belote and Baker, 1982). Sxl^{M1} is a semi-constitutive gain-of-function allele, which is lethal to males (Cline, 1979). The chromosome is marked with *y*, *sn* and *v*.

$tra2^{ts2}$ and $tra2^{ts1}$ are EMS alleles that cause *X/X* flies to develop as sterile females at permissive temperature (16 °C) and somatic males at restrictive temperature (29 °C; Belote and Baker, 1982). Heteroallelic combinations were used to increase the viability of homozygous mutants. *X/X* flies homozygous for the $tra2^{ts}$ alleles and carrying one copy of *pOtu-lacZ* were generated by the cross *y w ac/y w ac*; *cn* $tra2^{ts1}$ *bw/CyO X y w ac/*$B^s Y$; $tra2^{ts2}$ *bw/CyO*; *pOtu-lacZ/+*. At the restrictive temperature, pseudomales were identified as B^+ males. Flies were grown at 16 °C or 29 °C until eclosion. After eclosion, control and experimental

Table 1
tra-2 mutant pseudomale phenotypes

Cross	Genotype	Total gonads	Frequency of gonads (# of gonads)			p[a]
			Atrophic	Non-oogenic	Oogenic	
I. *tra2 ^{B}/tra2 ^{1} X/X pseudomales*						
A	*ovo + otu +*	125	0.23 (29)	0.46 (58)	0.30 (38)	
B	*ovo + otu −*	31	0.29 (9)	0.71 (22)	0 (0)	<0.005
C	*hs-otu*	71	0.45 (32)	0.15 (11)	0.39 (28)	0.10–0.05
D	*hs-otu/hs-otu*	36	0.17 (6)	0.33 (12)	0.50 (18)	<0.010
II. *Sibling tra2^{B}/tra2 ^{1} XX pseudomales*						
E1	*Sxl +* , *hs-otu*	35	0.51 (18)	0.20 (7)	0.29 (10)	0.90–0.10
E2	*Sxl ^{M1}*, no *hs-otu*	28	0.11 (3)	0.71 (20)	0.18 (5)	0.90–0.10
E3	*Sxl ^{M1}*, *hs-otu*	44	0.05 (2)	0.39 (15)	0.61 (27)	<0.005
III. *tra ^{4}/tra ^{1} XX pseudomales*[b]						
F1	no *hs-otu*	80	0.49 (39)	0.19 (15)	0.33 (26)	0.90–0.10
F2	*hs-otu*	41	0 (0)	0.12 (5)	0.88 (36)	<0.005

Cross A: +/+; *tra-2 ^{1}/CyO* X *+/B ^{s}Y*; *cn tra-2 ^{B} bw/CyO*. Cross B: *otu $^{P\Delta1}$ v f/FM6*; *tra-2 ^{1}/CyO* X *otu $^{P\Delta1}$ v f/B ^{s}Y*; *cn tra-2 ^{B} bw/CyO*. Cross C: +/+; *tra-2 ^{1}/CyO*; *hs-otu/hs-otu* X *+/B ^{s}Y*; *cn tra-2 ^{B} bw/CyO*; +/+. Cross D: +/+; *tra-2 ^{1}/CyO*; *hs-otu/hs-otu* X *+/B ^{s}Y*; *cn tra-2 ^{B} bw/CyO*; *hs-otu/hs-out*. Cross E: *y cm Sxl ^{M1} otu ^{2} v f/FM6*; *tra-2 ^{1}/CyO*; *hs-otu/TM3 Ser* X *+/B ^{s}Y*; *cn tra-2 ^{B} bw/CyO*; +/+. In this cross, *+/FM6*; *tra-2 ^{B}/tra-2 ^{1}*; *+/TM3 Ser* progeny displayed unexpectedly low viability and were not recovered in sufficient numbers for analysis. This possible interaction with the FM6 balancer may also explain the relatively high atrophic frequency in E1. Cross F: +/+; *tra ^{1}/TM6* X *FM6/Y*; *tra ^{4} kar ^{2} ry ^{5} red/TM6*. All crosses were cultured at 25 °C.

[a] Chi-square p-value comparing the oogenic gonads from each genotype with cross A as the expected. $p < 0.05$ considered statistically significant.

[b] Data from Hinson and Nagoshi (1999).

siblings were incubated for 3–7 days (if at 29 °C) or as long as 12 days (if at 16 °C). Their gonads were subsequently assayed for β-galactosidase activity.

2.3. Construction of dsx mutants

X/X or *X/Y* flies homozygous mutant for loss of function *dsx* alleles develop as somatic intersexes (Hildreth, 1965). Heteroallelic combinations were used to increase viability of *dsx* intersexes. *dsx ^{1}* is a null allele for both the male and female-specific *dsx* products. *dsx $^{M+R15}$* is a small deletion resulting in non-functional male and female DSX proteins. *dsx* intersexes with one copy of *pOtu-HA* were generated by the crosses: (a) *pOtu-HA/ +*;; *dsx ^{1} p ^{p}/TM6* X *+ /B ^{s}Y*;; *dsx $^{M+R15}$/TM6*. (b) *dsx $^{M+R15}$/TM6* X *pOtu-HA/B ^{s}Y*;; *dsx ^{1} p ^{p}/TM6*.

X/X animals carrying the dominant allele *dsx ^{D}* over a loss-of-function allele develop as somatic males (Gowen and Fung, 1957). Three amorphic alleles of *dsx*, *dsx $^{M+R15}$*, *dsx ^{1}* and *dsx ^{23}*, were used to generate *X/X* pseudomales. *dsx^{D} Sb e/dsx $^{-}$* pseudomales carrying one copy of *pOtu-lacZ* were generated by the crosses: (a) +/+; *pOtu-lacZ/ CyO*; *dsx ^{23} e/TM6* X *+/B ^{s}Y*;; *dsx ^{D}/TM6*. (b) *y w ac/FM6*; *pOtu-lacZ/ +* ; *dsx ^{1} p ^{p}/TM6* X *+/B ^{s}Y*; *pOtu-lacZ/ +* ; *dsx ^{D} Sb e/TM6*. (c) *y w ac/ +* ; *pOtu-lacZ/CyO*; *dsx $^{M+R15}$/ TM6* X *+/B ^{s}Y*;; *dsx ^{D} Sb e/TM6*.

2.4. Immunohistochemistry

Adults were aged 4–7 days after eclosion. Gonads were dissected in PBS (130 mM NaCl, 7 mM $Na_2HPO_4{\cdot}2H_20$, 3 mM $NaH_2PO_4{\cdot}2H_2O$). The tissues were fixed in a 1:1 solution of fix/heptane (fix: 4% paraformaldehyde in PBS) for 20 min with gentle agitation. Tissues were washed 4X in PBT (0.1% Triton X-100, 0.05% Tween 80 in PBS) for 15 min. The tissues were then permeabilized overnight in blocking buffer (PBT + 1 mg/ml crystalline bovine serum albumin, Sigma) at room temperature.

All antibodies were diluted to the appropriate concentration in blocking buffer. Incubations with primary antibodies were performed at 4 °C overnight. Primaries included the following monoclonals: anti-α-spectrin (3A9, 1:200 dilution; from the laboratory of D. Branton), anti-HTS-RC (HTS 655 4C, 1:200), from the laboratory of L. Cooley, anti-HA (1:200 dilution; Boehringer Mannheim), and a polyclonal VASA antibody preparation (1:1000) from the laboratory of L. and Y. Jan. Primaries were removed with three 15-minute washes in PBT, followed by incubation with secondary antibodies (diluted 1:200) for 3 h at room temperature. Secondaries used were Oregon Green or Texas Red-conjugated anti-mouse or anti-rabbit IgG (Molecular Probes). Nuclei were labeled with propidium iodide, 125 μg/ml RNase (Boehringer Mannheim) was added to the secondary antibody incubation solution. Preparations were mounted in Vectashield containing 1.5 μg/ml propidium iodide (Vector Laboratories).

2.5. β-Galactosidase staining

Construction of the *pOtu-lacZ* construct is described in Rodesch et al. (1995). The *ovo-lacZ* construct places β-galactosidase under the control of the *ovo* promoter (Mèvel-Ninio et al., 1995). To examine β-galactosidase expression in situ, gonads were dissected in PBS, then

incubated in 50% fixative/50% heptane in a covered depression slide with agitation for 20 min. The tissues were rinsed 3X in PBS + 0.1% Triton X-100. The tissues were incubated in staining solution overnight at 37 °C in the dark. After staining, the preparation was washed 2X for 20 min with PBS. The tissues were mounted in 50% glycerol in PBS. Stock solutions: solution A, 6.75 g/l NaCl, 6.63 g/l KCl, 0.66 g/l $MgSO_4 \cdot 7H_2O$, 0.54 g/l $MgCl_2 \cdot 6H_xO$; 0.33 g/l $CaCl_2 \cdot 2H_2O$; solution B, 1.4 g/l Na_2HPO_4, 0.1 g/l KH_2PO_4, 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 nine 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, M′-dimethylformamide, to a total volume of 1.0 ml in water.

2.6. 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 AHBT3 microscope using an Optronics LX450A camera for image capture. Figures were produced by transferring captured images to Adobe Photoshop 4.0. All processing was performed simultaneously over the entire image. Therefore, within each plate the relative signal intensities between different areas are as originally captured.

3. Results

3.1. tra2 expression in the soma is needed for otu germline promoter activity

Loss of function mutations in *tra2* cause *X/X* flies to develop as somatic males ('pseudomales') similar to that produced by *tra* mutant alleles. The sexually transformed male gonads ('pseudotestes') are much smaller than those found in wild-type males due to the arrested and aberrant differentiation of the *X/X* germ cells, which fail to undergo substantial male or female gametogenesis (Hinson and Nagoshi, 1999; Nöthiger et al., 1989). Pseudomales were produced using a *trans*-heterozygous combination of $tra2^{B}$ (an amorphic allele) and $tra2^{1}$ (a severe loss-of-function mutation), which displayed substantially higher viability than either homozygote, and had previously been reported to give rise to pseudomales indistinguishable from $tra2^{B}$ hemizygotes (Baker and Ridge, 1980; Mattox et al., 1996). *otu* promoter activity was monitored using the *pOtu-lacZ* transgene, which places the bacterial *lacZ* gene under *otu* promoter control. It is expressed in larval and pupal germ cells of both sexes, but becomes sex-specific in the adult gonad (Hinson et al., 1999; Rodesch et al., 1995). Substantial *otu* promoter activity is seen throughout oogenesis (Fig. 1A), while expression in mature testis is variable and limited to a few cells at the most apical tip (Fig. 1B, Hager and Cline, 1997; Hinson and Nagoshi, 1999).

We found that *pOtu-lacZ* was only infrequently expressed in the *X/X* germ cells of $tra2^{B}/tra2^{1}$ pseudotestes. Pseudomale gonads typically (19/21) showed no expression or only weak expression in a small subset of germ cells (Fig. 1C, D). In comparison, we tested the *ovo-lacZ* construct, which uses the promoter of the female germline-specific *ovo* gene and is only expressed in *X/X* (not *X/Y*) germ cells (Mèvel-Ninio et al., 1995; Oliver et al., 1994). In contrast to *pOtu-lacZ*, *ovo-lacZ* was expressed throughout the pseudomale gonad (10/10 gonads tested) indicating that germ cells are present and capable of germline-specific gene activity (Fig. 1E). These results indicate that *tra2* mutations have significant and specific effects on the germline expression of the *otu* promoter similar to that seen in pseudomales produced by mutations in *tra* (Hinson and Nagoshi, 1999).

3.2. tra2 can induce otu promoter activity after completion of sexual differentiation

We tested whether the *otu* promoter could be influenced by *tra2* expression after the differentiation of the somatic testis. If so, it would suggest a continuing and specific interaction with *otu* that is independent of earlier aspects of gonadal differentiation. In these experiments we used temperature-sensitive *tra2* alleles ($tra2^{ts2}/tra2^{ts1}$) that allowed us to modify the level of *tra2* activity in mature adults (Belote and Baker, 1982; Belote and Baker, 1987). *X/X* $tra2^{ts2}/tra2^{ts1}$ mutants grown at the permissive temperature (16–18 °C) develop as females and can produce mature egg chambers, though they remain sterile. If grown at 29 °C until eclosion, the same genotype will give rise to pseudomales similar to that seen with *tra2* null alleles.

First, we examined the expression of *pOtu-lacZ* in $tra2^{ts2}/tra2^{ts1}$ mutant flies grown at the restrictive temperature until eclosion, then incubated as adults at either 29 or 16 °C. If kept at 29 °C until the assay (3–7 days), no *pOtu-lacZ* expression was detected in the adult germline of all gonads ($n = 10$) tested (Fig. 2A). In contrast, if the $tra2^{ts}$ pseudomales were shifted to permissive conditions as adults (for 7–10 days), substantial *otu* promoter activity was observed (in 10/10 gonads; Fig. 2B). These results demonstrate that restoring *tra2* function after the completion of male sexual differentiation can induce *otu* promoter activity in the *X/X* germline, indicating continued sensitivity of *otu* to soma-dependent regulation.

We also performed the reciprocal experiment in which *X/X* $tra2^{ts}$ mutants were grown at the permissive temperature until eclosion, producing somatic females of wild-type morphology. These flies were then aged for varying amounts of time at either permissive or restrictive

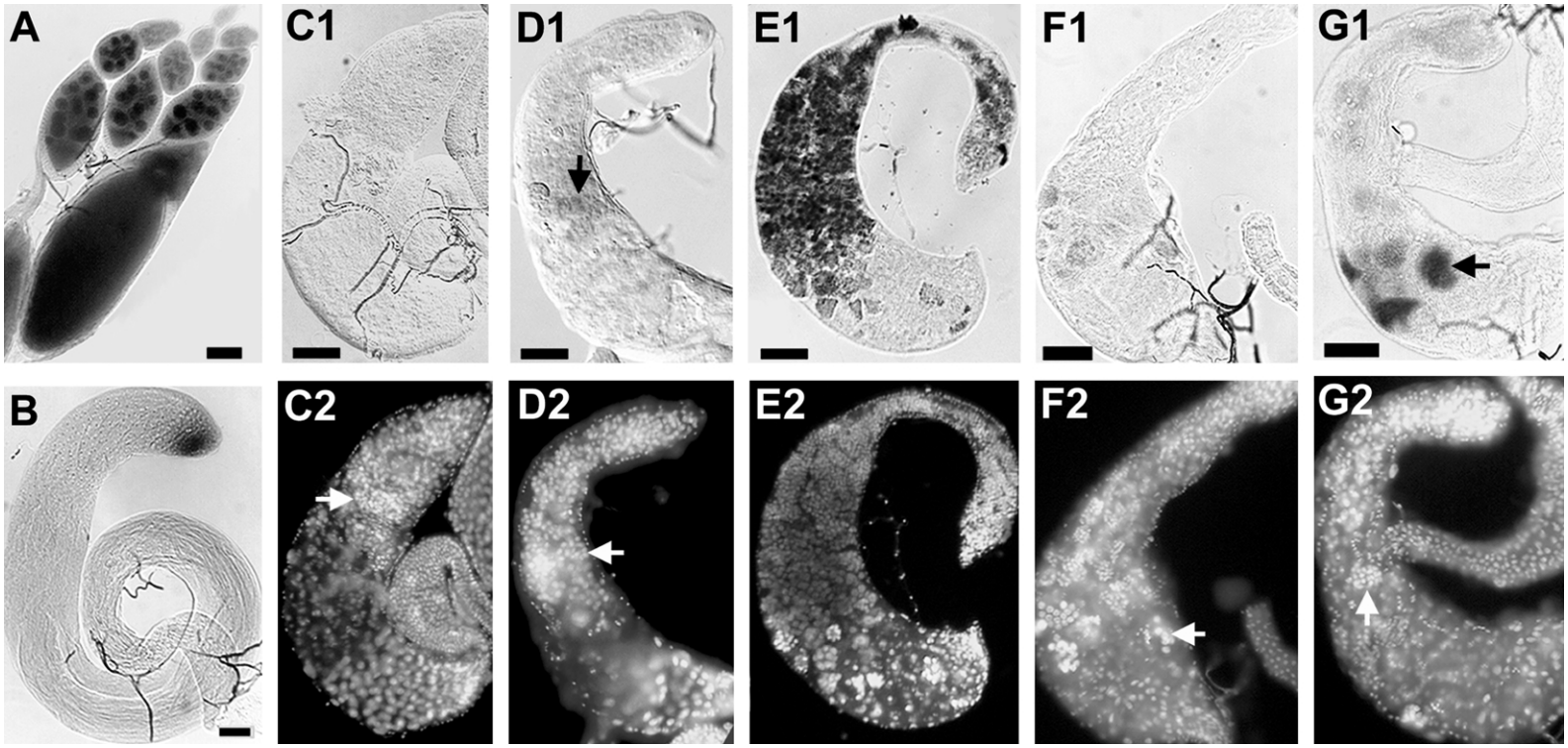

Fig. 1. *pOtu-lacZ* expression in adult gonads and in *X*/*X* pseudomales. Adult gonads were stained for β-galactosidase (dark stain) and DAPI (bright fluorescent labeling of nuclei). (A) Wild-type ovary with one copy of *pOtu-lacZ* expressing β-galactosidase in all germ cells. (B) Wild-type testis with one copy of *pOtu-lacZ* showing β-galactosidase staining localized to the apical tip. (C1–G1) Bright field micrographs of pseudomale gonads showing β-galactosidase staining pattern. (C2–G2) Fluorescent micrographs identifying DAPI labeling of gonads in C1–G1. DAPI identifies germ cell clusters within the gonad. Note that β-galactosidase staining tends to reduce DAPI fluorescence. (C, D) *X*/*X*; $tra2^{B}/tra2^{1}$ pseudomale gonads with one copy of *pOtu-lacZ*. C1 and D1 show little *pOtu-lacZ* activity despite many germ cell clusters. (E) *X*/*X*; $tra2^{B}/tra2^{1}$ pseudomale gonads with one copy of *ovo-lacZ*. Most, if not all, germ cell clusters express β-galactosidase. (F, G) *X*/*X*; dsx^{D}/dsx^{M+R15} pseudomale gonads with one copy of *pOtu-lacZ*. F1 and G1 show only sporadic *pOtu-lacZ* activity (arrows) despite many germ cell clusters. Size bar equals 50 μM.

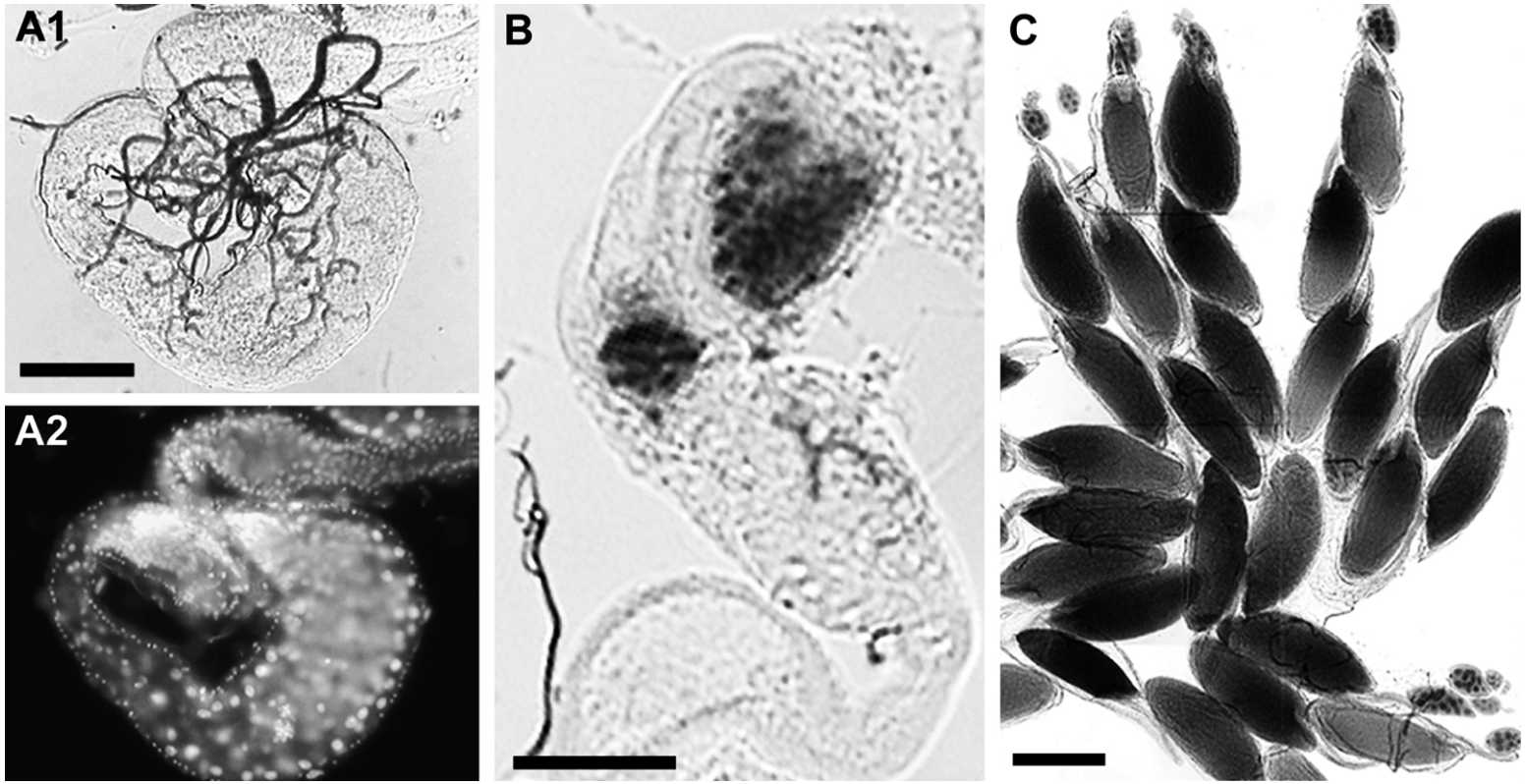

Fig. 2. *pOtu-lacZ* expression in adult gonads mutant for temperature-sensitive *tra2* alleles. Gonads are all from *X*/*X*; *tra2* ^{ts2}/*tra2* ^{ts1} adults and were stained for β-galactosidase activity (dark stain). (A) Typical mutant pseudotestis grown at 29 °C until eclosion and maintained at 29 °C until assayed. (A1) Bright field shows no β-galactosidase staining. (A2) DAPI stained fluorescent image of A1. (B) Typical mutant pseudotestis grown at 29 °C until eclosion and then incubated at 16 °C until assayed (7–10 days). Patches of β-galactosidase staining becomes evident. (C) Typical *X*/*X*; *tra2* ^{ts2}/*tra2* ^{ts1} ovary grown at 16 °C until eclosion and incubated at 29 °C until assayed (10 days). Extensive β-galactosidase expression is observed at all stages. Note retention of mature egg chambers that is indicative of the loss of *tra2* function. Size bar equals 100 μM.

conditions. As expected, if kept at 16 °C for 4–7 days post-eclosion, *pOtu-lacZ* was expressed in all ovaries ($n = 20$) at levels similar to that observed in wild-type females (data not shown). However, a shift to the restrictive temperature post-eclosion caused no noticeable decline in *pOtu-lacZ* expression (Fig. 2C). This was true even after 10 days at 29 °C when the ovaries displayed morphological aberrations consistent with the loss of *tra2* function, including the abnormal retention of mature chambers (Belote and Baker, 1982). These results suggest that *otu* promoter activity may not require continued *tra2* function after eclosion. However, we cannot discount the possibilities that sufficient *tra2* product remains even under restrictive conditions to keep the *otu* promoter active, or that β-galactosidase can perdure in the ovary for an unusually long period of time.

3.3. *dsx* is not required for *otu* expression in *X/X* germ cells

A substantial portion of the function of *tra2* (and *tra*) in somatic sexual differentiation occurs through the regulation of *dsx*. We therefore tested *dsx* mutations for their effect on *otu* germline expression. The *dsx* gene differs from *tra* and *tra2* in that it produces both male-specific (DSX^{male}) and female-specific (DSX^{female}) products that regulate the sexual differentiation of each sex (Baker and Ridge, 1980). If a *dsx* allele that constitutively expresses the male-specific *dsx* product (e.g. dsx^{D}) is made heterozygous with a loss-of-function mutation, *X/X* flies develop into somatic males similar to those produced by *tra* or *tra2* mutations (Baker and Ridge, 1980). We tested the effect of these *dsx* allele combinations on the expression of the *otu-lacZ* construct. Pseudomales were generated by the combination of dsx^{D} with the loss-of-function alleles, dsx^{1}, dsx^{23} or dsx^{M+R15}. At least eight gonads of each genotype were examined ($n = 25$ total) with identical results. The *otu* promoter was rarely expressed in the germline of *dsx* pseudomales, with expression limited to a few small germline clusters sporadically distributed among non-expressing cells (Fig. 1F, G). This reduction in β-galactosidase levels suggests that either a positive DSX^{female} or negative DSX^{male} somatic influence is acting on the germline-specific *otu* promoter in these *X/X* germ cells.

To distinguish between these possibilities, we examined the effect of *dsx* loss-of-function mutations that eliminate both male and female products. In the absence of *dsx* function, both *X/X* and *X/Y* flies develop as intersexes, in which sexually dimorphic tissues display intermediate sexual phenotypes. The chromosomally male and female intersexes have virtually identical external sexual morphology, though differ in the presence of certain sex-specific muscles and neurons (Taylor et al., 1994). The gonads of *dsx* mutant intersexes are very disorganized and small, with the germ cells largely undifferentiated and often degenerating. In these studies germ cells were identified by the expression of the germline-specific VASA protein or by the presence of spectrosomes or fusomes, while the *pOtu-HA* transgene was used to monitor *otu* promoter activity (Hay et al., 1988). *pOtu-HA* is a transgenic construct that produces an epitope-tagged OTU fusion protein controlled by the *otu* promoter and capable of rescuing *otu* null alleles to fertility (Hinson and Nagoshi, 1999).

In *X/X dsx* intersexes the germ cells displayed variable morphology, suggesting arrest at different stages in gametogenesis. Despite this phenotypic range, *pOtu-HA* was expressed in most, if not all, germ cells in all gonads examined ($n = 20$), indicating that *otu* promoter activity is not dependent on positive regulation mediated by DSX^{female} (Fig. 3A). When combined with the pseudomale results, where ectopic expression of the DSX^{male} product correlates with reduced *otu* activity in *X/X* germ cells, the data suggest that the male-specific *dsx* function may negatively regulate *otu* germline expression.

We tested this possibility by examining *X/Y* intersexes lacking *dsx* function. In all gonads examined ($n = 12$) OTU-HA was observed, as would be expected if in the absence of DSX^{male} the *otu* promoter became active even in chromosomally male germ cells. This again suggests that DSX^{male} inhibits *otu* promoter activity. However, the pattern of expression was less extensive than in *X/X dsx* intersexes, with clusters of expressing and non-expressing cells present in the same gonad (Figs. 3B and 4A, B). We found that this differential expression of OTU-HA correlated with the degree of gametogenic differentiation. OTU-HA-expressing germ cells displayed either spectrosomes or short, linear fusomes, suggesting a relatively immature and/or aberrant state of development (Fig. 4C). In comparison, germ cell clusters connected by large, multi-branched fusomes rarely, if ever, expressed the OTU-HA fusion protein (Fig. 4D). We conclude from these data that DSX^{female} is not required for *otu* promoter function while DSX^{male} has a negative effect, perhaps by influencing the differentiated state of the *X/X* germ cells.

3.4. Overexpression of both *otu* and *Sxl* stimulates gametogenesis in *tra2* pseudomales

The male transformation of *X/X* soma by the somatic sex regulatory genes blocks oogenic differentiation of *X/X* germ cells and may even cause the germline to initiate spermatogenic development (Nöthiger et al., 1989). In a previous study, we demonstrated that this gametogenic arrest in *tra* mutant pseudomales could be overcome by *hs-otu*, a construct in which *otu* is expressed from the heat shock *hsp-70* promoter (Hinson and Nagoshi, 1999). This suggests a *tra*-dependent somatic influence that, when mutated, reduces germline *otu* function to a level inadequate for oogenesis. We tested to see whether the same was true for *tra2*. Pseudomale gonads were categorized into three phenotypic classes defined by stage- and sex-specific molecular markers. The 'oogenic' gonads contained germ cells that initiated female differentiation as defined by the presence of ring canals containing the oogenesis-specific

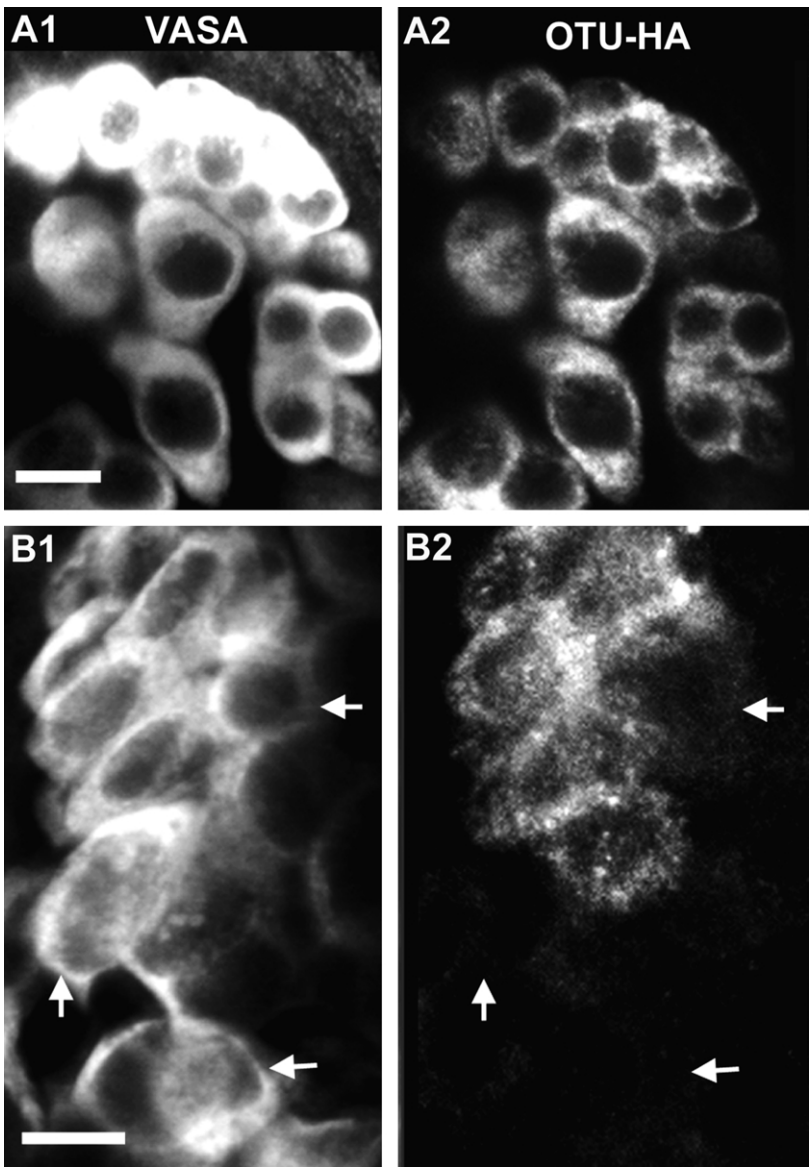

Fig. 3. *otu* promoter activity in *X/X* and *X/Y dsx* mutant intersexes. Gonads from *dsx* mutant intersexes carrying one copy of *pOtu-HA*. (A, B) Germ cells were co-labeled with anti-VASA (Texas Red) and anti-HA (Oregon Green) antibodies. (A1) Germ cells from *X/X*; dsx^{1}/dsx^{M+R15} gonad showing VASA-positive germ cells (Texas Red channel alone). (A2) Same preparation as A1 but showing OTU-HA expression (Oregon Green channel alone). All cells expressing VASA also express OTU-HA. (B) Germ cells from *X/Y*; dsx^{1}/dsx^{M+R15} gonad showing VASA-positive germ cells (Texas Red channel alone). (A2) Same preparation as B1 but showing OTU-HA expression (Oregon Green channel alone). Arrows point to subset of cells expressing VASA but not OTU-HA. Size bars equal 10 μM.

HTS-RC protein (Fig. 5A, E), a product of the *hu-li tai shao* gene (Robinson et al., 1994). The 'non-oogenic' class of gonads contained germ cells that initiated gametogenesis (as seen by the presence of spectrosomes and branched fusomes) but lack HTS-RC ring canals (Fig. 5B–D). We do not mean to imply by non-oogenic that these germ cells are sexually transformed, only that they lack clear evidence of female differentiation. The final 'atrophic' class represents gonads that do not contain VASA-positive cells.

Table 1 summarizes the results for the *tra2* pseudomale genotypes examined. The range of gonadal phenotypes produced approximated that seen with *tra* mutants (Hinson and Nagoshi, 1999), with 30% (38/125) of the gonads displaying some oogenic differentiation (Table 1, cross A). These gonads displayed only limited female germline development, as these contained few (<10) germ cell clusters expressing HTS-RC. To confirm that this molecular marker accurately identified oogenic differentiation in pseudomales, we tested whether HTS-RC expression in ring canals was dependent on *otu* function, which is required for early oogenic differentiation (Hinson et al., 1999; Oliver et al., 1990; Rodesch et al., 1997). As expected, a mutation in *otu* completely eliminated the oogenic class (Table 1, cross B).

The overexpression of *otu* in *tra2* pseudomales had only minor effects on the germline. The addition of one or two

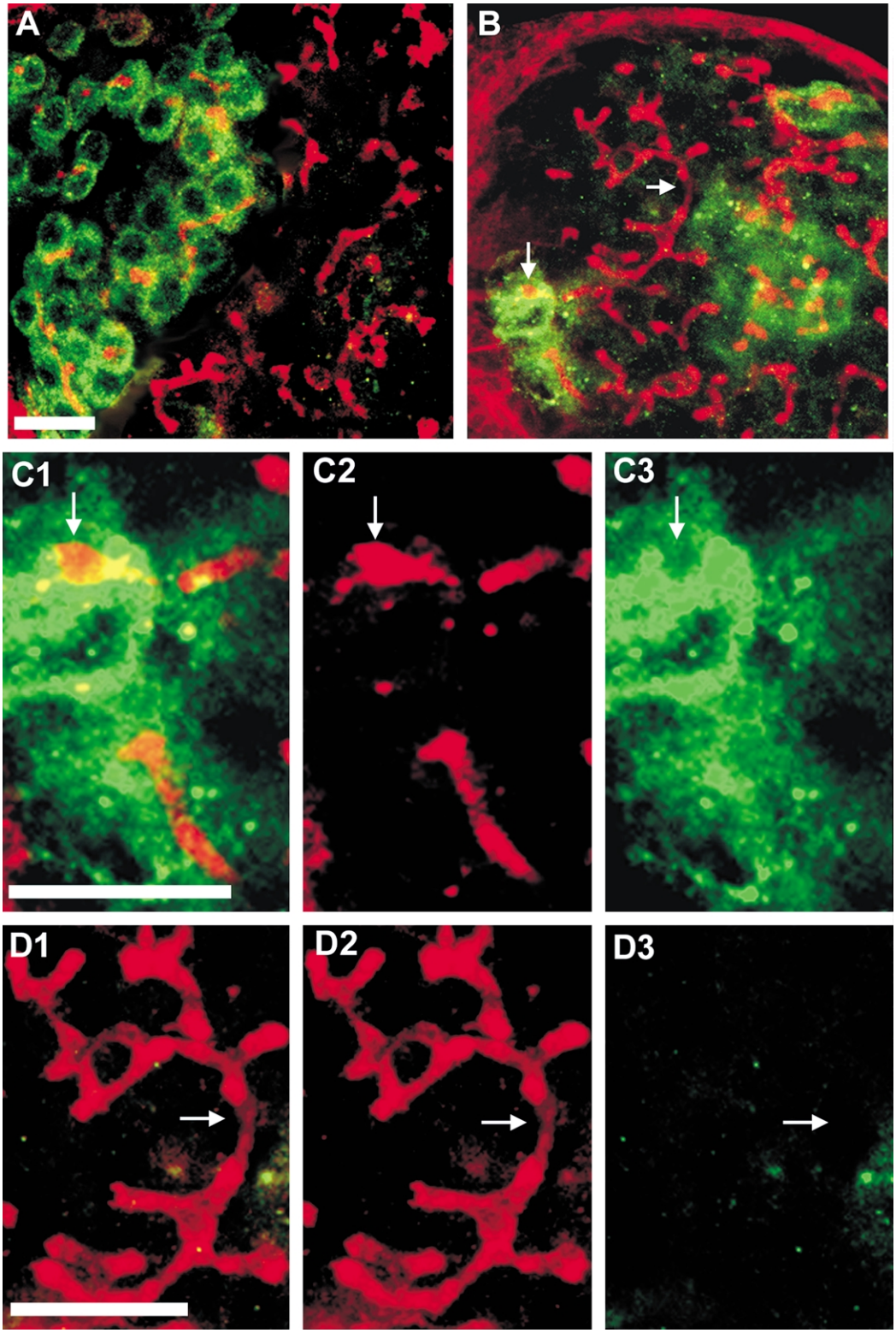

Fig. 4. *otu* promoter activity correlates with fusome development in the germline of *dsx* mutants. *X/Y*; dsx^{1}/dsx^{M+R15} gonads were labeled with anti-spectrin (Texas Red), which labels spectrosomes, fusomes, and cell periphery, and anti-HA (Oregon Green) antibodies. (A, B) Image of gonad showing only a subset of germ cells express cytoplasmic OTU-HA (green). Globular spectrosomes and linear or branched fusomes (red) identify the location of germ cells. Areas with large, branched fusomes do not appear to have much OTU-HA. (C1) Higher magnification image of region from B (indicated by vertical arrow) showing OTU-HA expression. (C2) Same as C1 but with Texas Red channel alone. Note that fusomes are short and linear. (C3) Same as C1 but with Oregon Green channel alone. (D1) Higher magnification image of region from B (indicated by horizontal arrow). (D2) Texas Red channel alone of D1 highlights large, multi-branched fusome. (D3) Same as D1 but with Oregon Green channel alone. Little OTU-HA expression is seen. Size bars equal 10 μM.

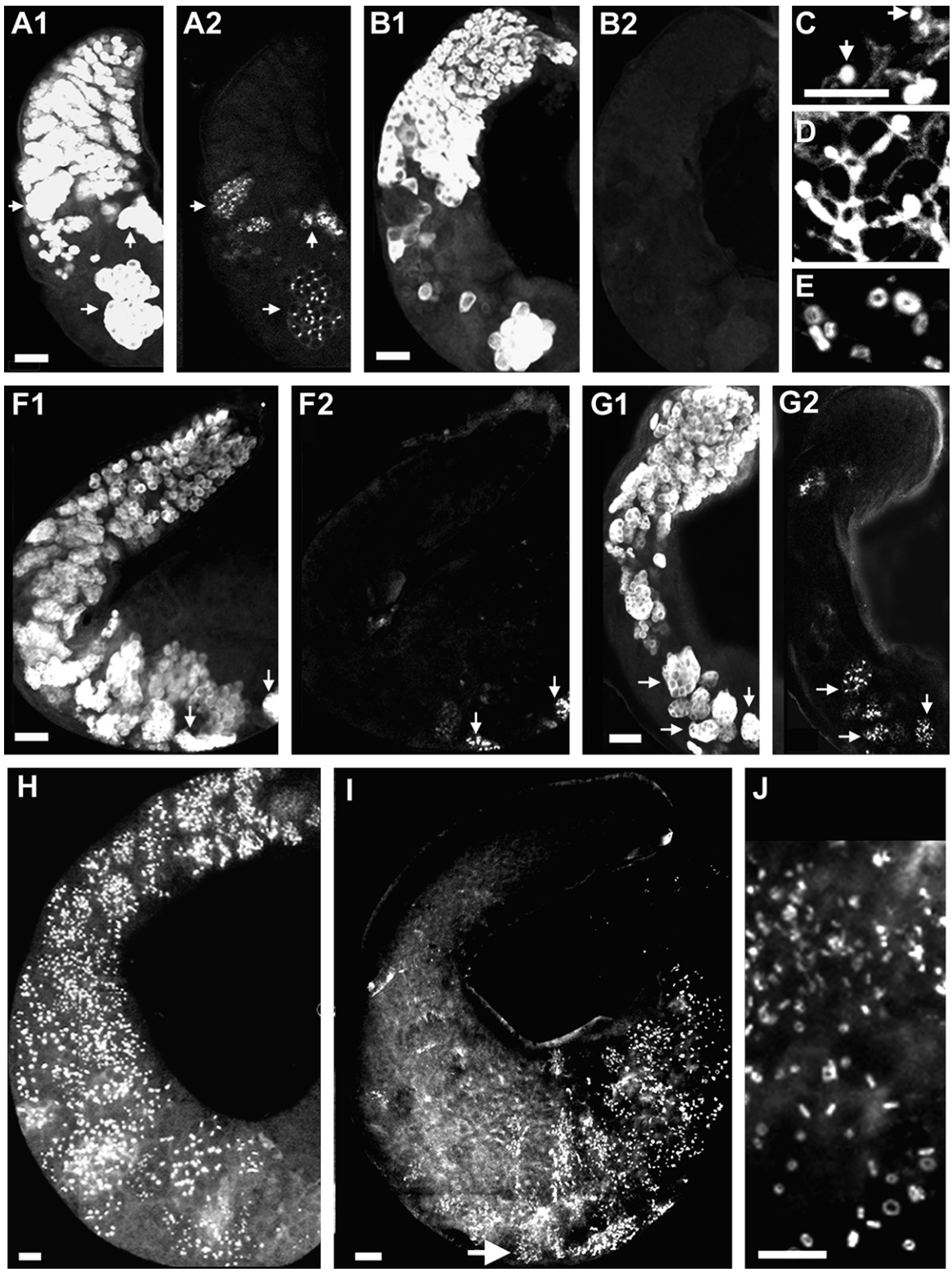

Fig. 5. The degree of oogenic differentiation in *X/X* pseudomales. (A, B) Gonads from *X/X*; *tra2* ^{B}*/tra2* ^{1} pseudomales labeled with anti-VASA (Texas Red) and anti-HTS-RC (Oregon Green) antibodies. (A) Oogenic pseudotestis containing clusters of germ cells connected by HTS-RC-positive ring canals (arrows). (A1) Texas Red channel alone showing VASA-positive germ cells. (A2) Same specimen as A1 but with Oregon Green channel alone. (B) Non-oogenic pseudotestis with no HTS-RC ring canals. (B1) Texas Red channel alone. (B2) Same specimen as A1 but with Oregon Green channel alone. Arrow points to VASA-positive germ cell cluster with no HTS-RC containing ring canals. (C) Spectrosome from non-oogenic pseudotestis labeled with anti-spectrin. (D) Fusome from non-oogenic pseudotestis labeled with anti-spectrin. (E) High magnification of HTS-RC-positive ring canals from oogenic pseudotestis. (F, G) Gonads from *X/X*; *tra2* ^{B}*/tra2* ^{1} pseudomales with (F) one copy of *hs-otu* or (G) one copy of *Sxl* ^{M1} labeled with anti-VASA (Texas Red) and anti-HTS-RC (Oregon Green)

copies of *hs-otu* raised the frequency of oogenic gonads from 30 to 39 and 50%, respectively (Table 1, crosses C, D). Though this latter induction was statistically significant ($p < 0.01$), it was modest relative to that observed in *tra* pseudomales carrying *hs-otu*, where the great majority (88%) of gonads became highly feminized (Table 1, cross F; Nagoshi et al., 1995). More importantly, in no case did we observe the same degree of feminization as caused by *hs-otu* in *tra* pseudotestes, where most germ cells underwent oogenic differentiation (Fig. 5H; Nagoshi et al., 1995). Instead, the oogenic *hs-otu*, *tra2* gonads contained only a few small clusters of HTS-RC-labeled ring canals (Fig. 5F1,2). Apparently the male sexual transformation induced by *tra* and *tra2* mutations differ in the sensitivities of the respective germlines to increased *otu* function.

This result may reflect the difference in the effect of *tra* and *tra2* mutations on germline *Sxl* expression. It was reported that SXL protein is present in *X/X* pseudomale germ cells produced by *tra* (or *dsx* mutations), but is reduced in the *tra2* pseudomale germline (Horabin et al., 1995). Therefore, the ability of *tra2* pseudomale germ cells to undergo oogenic differentiation in the presence of an ectopic *otu* source may be restricted due to insufficient germline *Sxl* function. This was tested by the introduction of Sxl^{M1}, a dominant *Sxl* allele that allows semi-constitutive gene expression in the germline (Bernstein and Cline, 1994). By itself, Sxl^{M1} had no significant influence on oogenic development in the *tra2* pseudomales. One copy of Sxl^{M1} did not increase the frequency of oogenic gonads (compared to Sxl^{+} pseudomales) and the gonads formed still contained only a few clusters of HTS-RC-labeled ring canals (Table 1, cross E; Fig. 5F, G). There is some indication that Sxl^{M1} may be suppressing the frequency of atrophic gonads, but we believe this is due to unspecified interactions with the X-chromosome balancer used (see Table 1 legend).

In comparison, substantial feminization was observed in sibling pseudomales carrying both *hs-otu* and Sxl^{M1}. A majority of the gonads (27/44) now contained oogenic germ cells (Table 1, E3). Even more compelling is that in most of the oogenic gonads (23/27) there was a dramatic increase in the number of feminized germ cell clusters, with HTS-RC-containing ring canals present throughout the gonad (Fig. 5I, J). This resulted in a 2–3-fold elongation in the length of the testis, similar to what we previously described for *tra* mutant pseudomales carrying one copy of *hs-otu* (Fig. 5H). We conclude that the *X/X* germ cells in *tra2* pseudomales can undergo oogenic differentiation if provided sufficient levels of both *Sxl* and *otu* functions.

4. Discussion

We demonstrated that the *dsx*-dependent genetic pathway is required to maintain the sex-specific expression pattern of *otu* in the adult germline. The results were most consistent with negative regulation from DSX^{male}, as the function of DSX^{female} is not required for *otu* expression in the *X/X* germline. However, this conclusion was only partially supported by studies with *dsx* mutant *X/Y* intersexes, as we found incomplete derepression of the *otu* promoter in *X/Y* germ cells lacking DSX^{male} (Fig. 3B). Furthermore, promoter activity appeared dependent on the differentiated state of the germ cell as defined by fusome morphology. Our favored interpretation of these results is that in the absence of *dsx* function, *X/Y* germ cells can initiate entry into either a male or female differentiation pathway. If the male decision is made, the *X/Y* germ cells form the normal large, branched fusomes and do not activate the *otu* promoter. In the event of the female choice, the *otu* promoter is active but an incompatibility between the feminized germ cells and their *X/Y* chromosomal constitution prevents normal fusome differentiation. Therefore, we postulate that the early expression from the *otu* promoter is dependent on the sexual state of the germ cells, which is influenced by the activity of the somatic *dsx* function.

This somatic influence on the germline can occur even after the maturation of the somatic gonad, as demonstrated by our finding that *X/X* germ cells remain sensitive to *tra2*-dependent activation of the *otu* promoter in the adult stage (Fig. 2). This supports our earlier proposal for continuous interactions occurring between the soma and germline with respect to *otu* activity in the mature ovary (Hinson and Nagoshi, 1999; Nagoshi et al., 1995).

An unexpected finding was that the germline of *tra-2* mutant *X/X* pseudomales could be induced to undergo further oogenic differentiation by the increased expression of both *Sxl* and *otu*, but not by either function alone (Table 1). This differs from the behavior of *tra* mutant pseudomale germ cells in which either *hs-otu* or a constitutive *Sxl* allele are sufficient to increase the feminization of the germline (Hinson and Nagoshi, 1999; Nagoshi et al., 1995; Nöthiger et al., 1989). Therefore, *tra-2* mutations have a more severe effect on germline differentiation than null alleles of *tra*. In combination with our results using *ovo-lacZ* (Fig. 1E), we conclude that the somatic interactions controlled by *tra* and *tra-2* can have specific and differing effects on the activity of different germline genes.

This is not the first indication that somatic male transformation caused by mutations in *tra* and *tra-2* might differ in their effects on the *X/X* germline. Horabin et al. (1995) reported that *tra-2* function in the soma, but not *tra*,

antibodies. Arrows point to HTS-RC containing cell clusters. (F1, G1) Texas Red channel alone showing germ cells. (F2, G2) Same preparations as F1 and G1 but with Oregon Green channel alone. (H) Gonad from *X/X*; tra^{4}/tra^{1} pseudomales with one copy of *hs-otu* labeled with anti-HTS-RC (Oregon Green). (I) Gonad from *X/Xx*; $tra2^{B}/tra2^{1}$ pseudomales with one copy of *hs-otu* and one copy of Sxl^{M1} labeled with anti-HTS-RC (Oregon Green). (J) Higher magnification of germ cell cluster (arrow in I). Arrows point to examples of ring canals. Size bars for C–E equal 10 μM. All others equal 50 μM.

is required for the germline expression of *Sxl*. However, other studies suggested that changes in *tra* activity might also affect the levels of germline *Sxl* (Hager and Cline, 1997; Nöthiger et al., 1989), hence it is not clear how qualitatively different *tra-2* and *tra* pseudomale germ cells are with respect to *Sxl* function. Nevertheless, if *tra-2* mutant pseudomale germ cells are more deficient in *Sxl* activity than their *tra* counterparts, it would readily explain why Sxl^{M1} is required in one but not the other for suppression by *hs-otu*.

In summary, our results demonstrate that sex-specific interactions with the soma are necessary to maintain germline expression of *otu* and *Sxl* at levels sufficient to support oogenic development. Given the essential and early functions played by *otu* and *Sxl* in the development of female germ cells, it is evident that the somatic sex-regulatory genes have critical roles in the determination and differentiation of germline sexual identity. In particular, our data suggest that consistent and optimal early expression of *otu* in *X/X* germ cells requires the absence of DSX^{male} activity in the soma acting in concert with sex-specific germline factors.

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