

The involvement of *ovarian tumour* in the intracellular localization of Sex-lethal protein

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Abstract

The *Drosophila ovarian tumour* gene is required at multiple times in the germline for oogenesis. A second gene, *Sex-lethal*, controls sex determination in the soma and also has a separate germline function affecting similar oogenic stages as *ovarian tumour*. We demonstrate that *ovarian tumour* is not required for early *Sex-lethal* gene expression in the female germline, as had been previously reported. Instead, we provide evidence that *ovarian tumour* has a specific role in the developmentally regulated accumulation of SEX-LETHAL protein within the cytoplasm and nucleus. Furthermore, the examination of nurse cell polytene chromosomes produced by certain ovarian tumour mutations showed that SEX-LETHAL protein can associate with discrete chromosomal sites in the germline and that this pattern appears to change as the egg chamber matures. This is the first indication that SEX-LETHAL is capable of direct physical interactions with chromosomes (albeit in a mutant background) and is consistent with the developmentally regulated nuclear localization of SEX-LETHAL being important for oogenesis.

Keywords: oogenesis, *transformer-2*, *ovarian tumour*, *transformer*, *Sex-lethal*

Introduction

The *ovarian tumour* (*otu*) gene is required during several stages of female germline development (King & Riley, 1982; Storto & King, 1988). During normal oogenesis, germline stem cells 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 & 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 branched derivative of the spectrosome (Lin & 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 tumours' in which egg chambers are filled with hundreds of poorly differentiated germ cells (Geyer *et al.*, 1993; Rodesch *et al.*, 1995; Storto & King, 1988). Severe hypomorphic *otu* mutations produce larger ovarian tumours associated with arrest during later cystocyte divisions, while weaker alleles allow the formation of differentiated egg chambers arrested during late vitellogenic stages (King *et al.*, 1986; King & Riley, 1982; Rodesch *et al.*, 1997).

Certain mutant alleles of *Sex-lethal* (*Sxl*) can also cause ovarian tumours similar to those produced by *otu* mutations, indicating an early role in oogenesis (Perrimon *et al.*, 1986; Salz *et al.*, 1987). In the soma, *Sxl* controls sex determination by acting as a sex-specific regulator of RNA splicing (Bell *et al.*, 1988; Inoue *et al.*, 1990; Sakamoto *et al.*, 1992; Valcárcel *et al.*, 1993) and translation efficiency (Gebauer *et al.*, 1998; Kelley *et al.*, 1997). It is not known which or if either of these functions is involved in the regulation of oogenesis.

The germline-specific SXL protein displays a complex and dynamic pattern of expression and subcellular localization during oogenesis (Bopp *et al.*, 1993). It is initially found in the cytoplasm of *X/X* stem cells and continues to accumulate until the first cystoblast division (which forms two cystocytes). This is followed by a precipitous decrease in detectable SXL levels during the subsequent three cystocyte divisions. Shortly after the formation of the 16-cell cyst, SXL re-accumulates, but this time in the nucleus. Additional intracellular shuffling of SXL protein occurs in postgermarial stages (Bopp *et al.*, 1993). Given the somatic examples

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of *Sxl* functioning in both the nucleus and cytoplasm, it is likely that this germline distribution pattern is important for the regulation of oogenesis.

Regulatory and functional interactions have been observed between *Sxl* and *otu*. Mutations in *otu* were reported to prevent the early expression of SXL protein and disrupt female-specific *Sxl* RNA processing, leading to suggestions that *otu* might regulate *Sxl* gene expression (Bopp *et al.*, 1993; Oliver *et al.*, 1993; Pauli *et al.*, 1993). In addition, dominant, semiconstitutive *Sxl* alleles can improve the phenotypes of certain *otu* mutant combinations, leading to suggestions that *Sxl* may act downstream of *otu* in a germline genetic pathway controlling oogenesis (Bae *et al.*, 1994; Pauli *et al.*, 1993). However, this *Sxl*-induced rescue is incomplete and often allele-specific, complicating interpretations of these data. How *otu* might interact with *Sxl* has not been determined.

In this paper, we investigate how *otu* acts with *Sxl* to control oogenesis. Our studies demonstrate that *otu* is not necessary for early *Sxl* expression. Instead, *otu* mutations can arrest germ cell development at stages with different patterns of SXL localization, including at least one early period when SXL does not normally accumulate. Furthermore, this interaction between *otu* and *Sxl* appears specific, as conditions that allow *otu* mutant germ cells to undergo further oogenic differentiation do not alleviate the disruption of SXL localization. Finally, we show that SXL can bind to specific chromosomal sites in a pattern that appears to vary as the germ cells differentiate. This suggests that the regulated nuclear localization of SXL product is an essential regulator of early oogenesis.

Results

Two otu-dependent Sxl expression patterns are observed during early oogenesis

It was previously reported that the tumour-susceptible germ cells produced by *otu* mutations in females contained aberrantly high levels of the male-specific (non-functional) *Sxl* RNA splice form, leading to lower than normal amounts

of SXL protein and the conclusion that *otu* is required for regulation of *Sxl* RNA splicing (Bopp *et al.*, 1993; Oliver *et al.*, 1993; Pauli *et al.*, 1993). However, a complication of these studies is their dependence on partially functional *otu* alleles, which we have previously demonstrated to block oogenesis at a later stage than the null condition (Rodesch *et al.*, 1997). Hence the effect of the absence of *otu* function on *Sxl* expression could only be inferred. To directly characterize the null phenotype we examined germ cells mutant for the *otu*^{PΔ1} allele, a deletion of the entire *otu* coding region.

Homozygous *otu*^{PΔ1} adult ovaries form quiescent ovarioles lacking egg chambers or, less frequently, small tumorous chambers (Geyer *et al.*, 1993). In both phenotypes, clusters of poorly differentiated germ cells are present that can be unambiguously identified by germline-specific markers (Rodesch *et al.*, 1995). In contrast to reports on hypomorphic *otu* alleles, we found that SXL protein was typically expressed and accumulated in the cytoplasm of the mutant germ cells (Fig. 1A). These results indicate that at least the initial expression of SXL protein in the germline is not dependent on *otu* function.

To reconcile our results with past studies, we re-examined the SXL distribution pattern in *otu*^{PΔ2} mutants, a hypomorphic allele previously reported to greatly reduce SXL protein levels (Bopp *et al.*, 1993). In agreement with these studies, SXL expression was reduced or absent in the germ cells of the tumorous chambers that make up the bulk of the *otu*^{PΔ3} mutant ovary (Fig. 1B). However, we consistently found cytoplasmic SXL in the germ cells of the germarium (Figs 1–2). This was particularly evident in young ovaries (1–2 days posteclosion) where germaria were most prominent. As the mutant ovaries aged, there was a reduction in germarium size, making their detection and analysis difficult (data not shown). To confirm that the SXL-expressing cells were of germline origin, we demonstrated that they were connected by fusomes, a germline-specific structure (Fig. 1C1). Therefore, *otu*^{PΔ3} does not prevent the initial expression of SXL in the germline as previously reported, but blocks oogenesis at a stage when SXL is mostly absent.

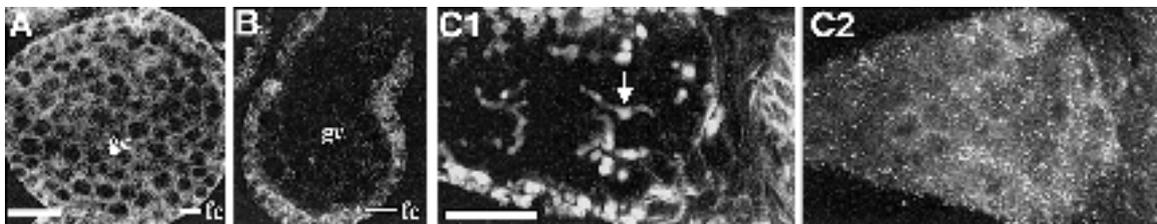


Figure 1. SXL distribution in *otu* mutant X/X ovaries. All preparations were treated with anti-SXL antibodies (Oregon Green). (A) An *otu*^{PΔ1} tumorous chamber showing germ cells and follicle cells expressing substantial levels of cytoplasmic SXL protein (cytoplasmic SXL is observed in follicle cells during normal oogenesis, Bopp *et al.*, 1993). (B) *otu*^{PΔ3} tumorous chamber showing reduced levels of cytoplasmic SXL in the germ cells. (C) *otu*^{PΔ3} mutant germarium labelled with anti-SXL (Oregon Green) and polyclonal antispectrin (Texas Red). (C1) View with Texas Red channel alone showing fusomes (arrow). (C2) Oregon Green channel alone showing expression of SXL in germ cells. Bars = 10 μm.

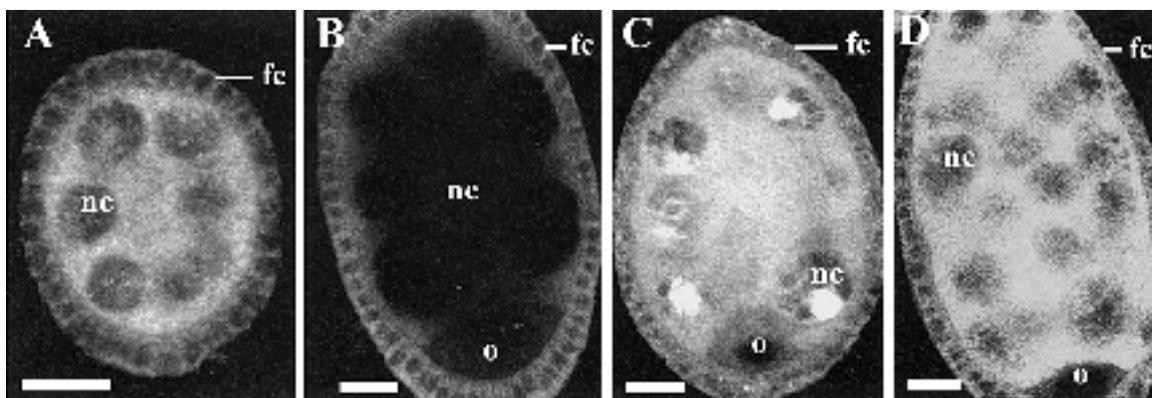


Figure 2. SXL distribution in *Sxl^{M1}* and *otu* mutant *X/X* ovaries. Preparations were labelled with anti-SXL (Oregon Green) and viewed by confocal microscopy. (A–C) Germarium and chambers from *Sxl^{M1} otu²/Sxl⁺ otu⁺* ovaries. These display the wild-type pattern of SXL distribution. (A) High cytoplasmic SXL labelling observed in the germline stem cells and cystoblasts (sc/cb) and greatly reduced levels in the dividing cystocytes (arrow). (B) A stage 4 previtellogenic chamber showing cytoplasmic SXL protein in both nurse cells (nc) and follicle cells (fc). (C) A stage 8 vitellogenic egg chamber with little cytoplasmic SXL protein in the nurse cells (nc). Compare with the relatively high cytoplasmic SXL in the surrounding follicle cells (fc). The oocyte (o) is at the posterior end and expanding. (D–F) Germarium and chambers from *y cv otu^{PΔ5}/y cv otu^{PΔ5}* ovaries. (D) Germarium showing high levels of cytoplasmic SXL in stem cells and cystoblasts (sc/cb) with some cytoplasmic accumulation and nuclear localization in the cystocytes (arrow). (E) A previtellogenic chamber with high levels of diffusely distributed cytoplasmic SXL in the nurse cells. The large nurse cell nuclei display more localized SXL (arrow). (F) A stage 8 chamber with high levels of cytoplasmic and localized nuclear (arrow) SXL protein in the nurse cells (nc), but not in oocyte (o). (G–I) Germarium and chambers from *Sxl^{M1} otu²/Sxl⁺ otu^{PΔ3}* ovaries. (G) Germarium showing high levels of cytoplasmic SXL in stem cells and cystoblasts (sc/cb) and in dividing cystocytes (arrow). The germarium of this genotype is typically extended. (E) A previtellogenic chamber with high levels of cytoplasmic SXL in all germ cells. (F) A stage 8 chamber with high levels of cytoplasmic SXL protein in the nurse cells (nc), but not in oocyte (o). Bars = 5 μm.

These results are consistent with our previous descriptions of the *otu^{PΔ1}* and *otu^{PΔ3}* mutant phenotypes. The *otu^{PΔ1}* mutation disrupts oogenesis prior to or during the first cystoblast division (Rodesch *et al.*, 1997) when cytoplasmic SXL levels are normally relatively high (Bopp *et al.*, 1993; Fig. 2A). In comparison, *otu^{PΔ3}* mutant germ cells show aborted development during the later cystocyte divisions (Rodesch *et al.*, 1997), when cytoplasmic SXL levels markedly decline (Bopp *et al.*, 1993; Fig. 2A). Therefore, *otu* is required during two early developmental stages that can be defined by distinctive SXL expression and localization patterns.

The involvement of otu on SXL distribution during later oogenic stages

In wild-type ovaries, SXL protein accumulates in the nurse cell cytoplasm prior to vitellogenesis, but decreases significantly by stage 8 (Bopp *et al.*, 1993; Fig. 2B,C). The maturation of egg chambers to vitellogenic stages can be determined by several phenotypes, including the elongated shape of the egg chamber, the presence of yolk in the oocyte, and the expression pattern of the *staufen* gene product (STAU), which accumulates in the oocyte beginning at stage 8 and subsequently becomes anchored to the posterior pole by stage 10 (Clark *et al.*, 1994; Giniger *et al.*, 1993; St. Johnson *et al.*, 1991; Fig. 3A). By these criteria, ovaries mutant for the hypomorphic *otu^{PΔ5}* allele contain chambers typically reaching stages 8–9, and on rare occasions stage 10 (Fig. 3B,C). Despite this degree of egg maturation, the *otu^{PΔ5}* mutant germ cells displayed high SXL

levels in the cytoplasm as well as in portions of the nucleus, a pattern more consistent with previtellogenic nurse cells (Fig. 2E,F, compare with Fig. 2B).

These results indicate that not only does the effect of *otu* mutations on SXL localization persist into later oogenic stages, but it can also be separated from other aspects of egg chamber development. This suggests a specific interaction between the two genes. We tested this possibility by using a hypomorphic *otu* allele combination that can be partially suppressed by dominant, semiconstitutive alleles of *Sxl* (Bae *et al.*, 1994; Pauli *et al.*, 1993). If the interaction between *otu* and *Sxl* is specific, then the improvement in the ovarian phenotype of chambers still mutant for *otu* should not affect the aberrant SXL pattern.

The *otu^{PΔ3}* allele is a deletion in the promoter region of *otu* that greatly reduces, but does not eliminate, *otu* function (Geyer *et al.*, 1993). When *otu^{PΔ3}* is made heterozygous with the severe *otu²* allele, tumorous chambers are produced, along with a few (10–20%) more mature eggs (Bae *et al.*, 1994). The tumorous phenotype was associated with high levels of SXL in germ cell cytoplasm, similar to that observed with *otu^{PΔ1}* mutants (data not shown). In comparison, females mutant for the same *otu* alleles, but now carrying one copy of the semiconstitutive *Sxl^{M1}* allele (*Sxl^{M1} otu²/Sxl⁺ otu^{PΔ3}*), produced primarily (80–90%) egg chambers with differentiated nurse cells, and oocytes based on morphology and the incorporation of stage-specific components of the ring canals (Bae *et al.*, 1994). In this genotype, STAU protein was expressed in the oocyte of the more mature mutant chambers, but was not

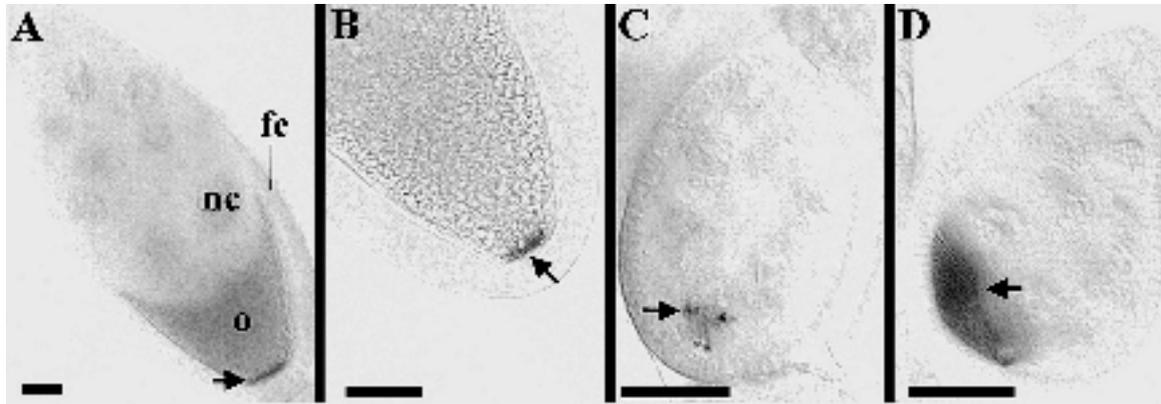


Figure 3. STAU expression in mutant vitellogenic egg chambers. (A–D) Chambers were labelled with anti-STAU antibodies tagged with alkaline phosphatase (dark stain). (A) Wild-type stage 10 chamber with STAU anchored at the posterior end of the oocyte (o, arrow). No expression is seen in nurse cells (nc) or follicle cells (fc) at this stage. (B) Most mature (and relatively rare) *otu*^{PΔ5}/*otu*^{PΔ5} chamber showing STAU anchored at posterior end of oocyte. (C) *otu*^{PΔ5}/*otu*^{PΔ5} chamber showing STAU expressed but not anchored at posterior end of oocyte. (D) *Sxl*^{M1} *otu*²/*Sxl*⁺ *otu*^{PΔ5} chambers showing expression and oocyte localization of STAU (arrows). However, posterior anchoring does not occur. Bars = 10 μm.

anchored to the posterior pole (Fig. 3D), providing physiological evidence for at least partial maturation to stage 8 and arrest shortly thereafter.

Once again, this improvement in oogenic differentiation did not alleviate the aberrant SXL distribution pattern. Cytoplasmic SXL remained high in the germ cells of all stages, including those in vitellogenic chambers (Fig. 2H,I). Importantly, this was also the case for the germarium, which in the suppressed genotype was typically abnormally elongated (Fig. 2G). The germarium contained apical cells with high levels of cytoplasmic SXL, indicative of stem cells and cystoblasts, as well as persistent SXL accumulation in the more distal dividing cystocytes. A similar result was often observed in *otu*^{PΔ5} germaria, although more variably as would be expected with weak hypomorphic alleles (Fig. 2D). In comparison, when *Sxl*^{M1} was present in an *otu*⁺ background, SXL cytoplasmic levels were markedly reduced in the dividing cystocytes (Fig. 2A). Therefore, even in a genotype capable of vitellogenic differentiation, the germ cells were abnormal with respect to SXL localization both at early germarial and later vitellogenic developmental stages. Taken together, these results demonstrate that the germline distribution pattern of SXL protein reflects the level of *otu* activity present, regardless of the state of germ cell differentiation.

SXL associates with discrete chromosomal sites in the developing germline

In our studies with *otu*^{PΔ5} we consistently observed abnormally prominent foci of nuclear SXL localization (Fig. 2G–I). *otu*^{PΔ5} is one of several hypomorphic *otu* alleles that cause the formation of polytene chromosomes in the mutant nurse cells (King *et al.*, 1981), suggesting the possibility that the SXL foci may be due to this more organized chromosomal arrangement. We examined this nuclear distribution of SXL in more detail. In the nurse cells

of previtellogenic chambers, we found SXL protein concentrated within a narrow band at what appears to be a single chromosomal location (Fig. 4A,B). In more elongated chambers (indicating differentiation to stage 7), SXL is associated with multiple chromosomal sites, as well as in a spherical concentration that might be extrachromosomal (Fig. 4C,D). Therefore, SXL may undergo stage-specific changes in its localization within the nucleus.

Sensitivity of SXL localization to increased levels of otu

To further study the correlation between *otu* activity and SXL protein distribution, we examined X/X pseudomales produced by mutations in the somatic sex regulatory gene *transformer-2* (*tra-2*). Loss of *tra-2* function in X/X flies results in somatic male differentiation, producing pseudotestes that are reduced in size and contain X/X germ cells arrested early in gametogenesis (Nöthiger *et al.*, 1989). The germ cells are of variable phenotypes, but can be readily identified by the expression of the germline-specific VASA product (Fig. 5A). This provides an opportunity to examine the behaviour of female germ cells blocked early in development, but that are genetically wild-type for *otu* and the other germline-specific genes regulating oogenesis.

It was previously reported that *tra-2* expression is required for the accumulation of SXL protein in the female germline (Horabin *et al.*, 1995). However, we found that this requirement is not absolute, as 60% of the *tra-2* mutant gonads examined contained at least one cluster of germ cells with detectable levels of SXL protein (Table 1A). These almost always (in 17/18 gonads) displayed cytoplasmic SXL that co-localizes with the germline-specific VASA protein (Fig. 5A). This pattern is consistent with arrest prior to the cystocyte divisions.

The levels of both *Sxl* and *otu* were simultaneously increased by the introduction of *Sxl*^{M1} and a heat-shock

Figure 4. Localization of SXL protein on polytene chromosomes in the germline. Ovaries from *X/X* flies homozygous mutant for *otu*^{PA5} were labelled with anti-SXL (Oregon Green) antibodies and propidium iodide, which stains DNA and fluoresces in the Texas Red channel. (A) Previtellogenic chamber showing nurse cells with substantial cytoplasmic SXL as well as a localized concentration on the polytene nurse cell chromosome (arrows). (B1) Merged image and high magnification view of section in (A). (B2) Oregon Green channel alone. (C) Vitellogenic chamber with SXL in cytoplasm and nuclei of the nurse cells. (D1) Merged image and high magnification view showing multiple chromosomal sites and one possible extrachromosomal site. (D2) Oregon Green channel alone. Bars = 10 µm.

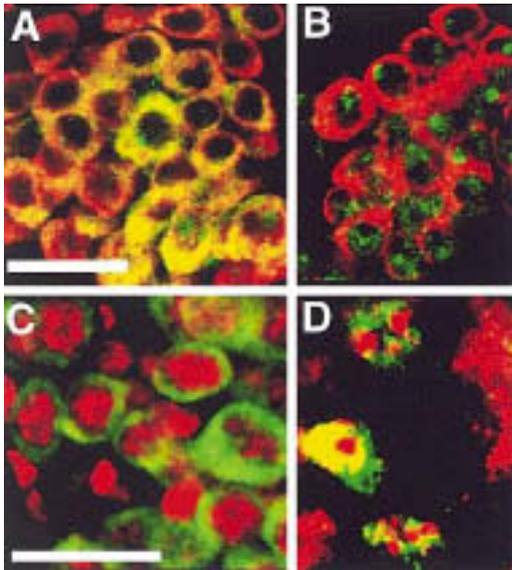
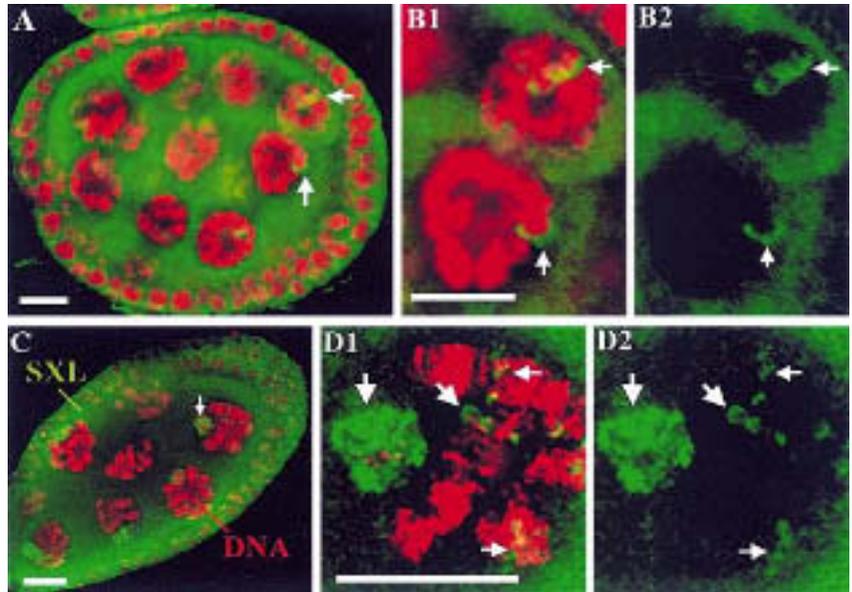


Figure 5. SXL protein localization in pseudomale germ cells. (A, B) Germ cells were labelled with anti-VASA (Texas Red) and anti-SXL (Oregon Green) antibodies. (A) Germ cells from *X/X; tra2^B/tra2^I* pseudomale showing SXL predominantly in the cytoplasm of VASA-positive germ cells. VASA is also found in the cytoplasm. (B) Germ cells from *X/X; tra2^B/tra2^I* pseudomale with *hs-otu* and *Sxl^{M1}*. SXL is now predominantly found in the nucleus. (C, D) Germ cells were labelled with anti-SXL (Oregon Green) antibodies and propidium iodide, which stains DNA and fluoresces in the Texas Red channel. (C) Germ cells from *X/X; tra⁴/tra¹* pseudomale showing predominantly cytoplasmic SXL. (D) Germ cells from *X/X; tra⁴/tra¹* pseudomale with one copy of *hs-otu* showing SXL localization to the nucleus. A and B are at same magnification, as are C and D. Bars = 10 µm.

inducible *otu* transgene (*hs-otu*) to *tra-2* mutant pseudomales. This resulted in a substantial increase in testis size due to the further differentiation of the germ cells (Hinson & Nagoshi, in press). This phenotypic change correlated with an increase in SXL accumulation such that 95% of

gonads now contained SXL-expressing germ cells, with most now showing nuclear localization of the protein (Table 1B3, Fig. 5B). In contrast, *Sxl^{M1}* or *hs-otu* alone did not significantly alter the pseudomale phenotype and resulted in only a modest increase in nuclear localization, to 15% and 20% of gonads, respectively (Table 1B1–B2; Hinson & Nagoshi, in press).

A similar result was observed in the germline of *X/X* pseudomales produced by mutations in another somatic sex regulatory gene, *transformer* (*tra*). In the presence of *otu*⁺ only 12% of the pseudomale gonads contained germ cells with predominantly nuclear SXL localization (Table 1C1). The majority displayed no SXL expression, reflecting the high frequency of gonads lacking germ cells produced by this *tra* allele combination, with most of the remainder displaying cytoplasmic accumulation (Fig. 5C). We previously showed that in the case of *tra* mutants, the addition of one copy of *hs-otu* alone was sufficient to cause an increase in oogenic differentiation (Hinson & Nagoshi, 1999; Nagoshi *et al.*, 1995). This change in phenotype correlated with a high frequency of nuclear SXL localization in the germline (Table 1-II), and a close association of SXL protein with the chromatin of these cells (Fig. 5D). Taken together, we conclude that despite the different methods of arresting germ cell development, these results are consistent with those from studies on *otu* mutant ovaries. Changes in *otu* expression influence the pattern of cytoplasmic and nuclear localization of SXL protein within the *X/X* germline.

Discussion

Mutations in *otu* can block early oogenic differentiation at two stages which are distinguishable by their intracellular SXL protein distributions. In the absence of *otu* function,

Table 1. SXL protein distribution in X/X pseudomales

Cross	Genotype	Total gonads	Frequency of gonads with germ cells expressing:			P§
			No SXL*	Cytoplasmic SXL†	Reduced cytoplasmic with nuclear SXL‡	
I. <i>tra2^B/tra 2</i> pseudomales:						
A	<i>Sxl⁺ otu⁺</i>	30	0.40 (12)	0.57 (17)	0.03 (1)	
B1	<i>Sxl^{M1} otu⁺</i>	20	0.35 (7)	0.50 (10)	0.15 (3)	0.90–0.10
B2	<i>Sxl⁺ hs-otu</i>	25	0.52 (13)	0.28 (7)	0.20 (5)	0.90–0.10
B3	<i>Sxl^{M1} hs-otu</i>	20	0.05 (1)	0.25 (5)	0.70 (14)	< 0.01
II. <i>tra^A/tra¹</i> pseudomales:						
C1	<i>Sxl⁺ otu⁺</i>	25	0.64 (16)	0.24 (6)	0.12 (3)	< 0.025
C2	<i>Sxl⁺ hs-otu</i>	25	0	0.16 (4)	0.84 (21)	< 0.005¶

Cross A: *+/+*; *tra-2^B/CyO X+/B^S Y*; *cn tra-2^B bw/CyO*. Cross B: *y cm Sxl^{M1} otu² v f/FM6*; *tra-2¹/CyO*; *hs-otu/TM3 Ser X⁺/B^S Y*; *cn tra-2^B bw/CyO*; *+/+*. Cross C: *otu^{PA1} v f/FM6*; *tra-2¹/CyO X otu^{PA1} v f/B^S Y*; *cn tra-2^B bw/CyO*. Cross C: *+/+*; *tra¹/TM6 X FM6/Y*; *tra^A kar² ry⁵ red/TM6*.

*No germ cells with detectable SXL.

†All SXL-expressing cells show predominantly cytoplasmic localization.

‡Contains at least one cluster of germ cells with predominantly nuclear SXL localization.

§ χ^2 probability using Cross A as the expected.

¶The same χ^2 probability was found for the comparison with Cross C1.

the mutant germ cells display a cytoplasmic SXL accumulation which is consistent with morphological criteria indicating arrest at or before the first cystocyte division, while a hypomorphic *otu* allele that disrupts later cystocyte divisions results in germ cells with little cytoplasmic SXL. Similar results were obtained from experiments using X/X pseudomales. In this case, female germline development was arrested not by *otu* mutations, but by a male transformation of the soma that disrupts essential, but largely uncharacterized, soma–germline interactions. Yet even in this markedly different and non-ovarian background, increased *otu* activity was still necessary (and in the case of *tra* pseudomales sufficient) for germ cells to reach a state where SXL becomes nuclear rather than cytoplasmic.

We considered the possibility that the aberrant SXL localization phenotype is a non-specific and secondary consequence of the arrested development caused by the *otu* mutations. If correct, then the SXL distribution pattern should correspond to the differentiated state of the arrested germ cell, such that manipulations suppressing the developmental arrest will lead to a more normal SXL expression pattern. However, this was not observed in experiments using hypomorphic and suppressed *otu* allele combinations. In each case, despite the mature morphology and physiology of the egg chambers with respect to ring canals, nurse cell differentiation, yolk protein accumulation, and the transport of STAU to the oocyte, the intracellular distribution of SXL remained aberrant and characteristic of less mature stages (Fig. 2). Therefore, the SXL distribution pattern is more dependent on the level of *otu* activity than the differentiated state of the germ cell or egg chamber.

Additional evidence comes from the *tra-2* mutant pseudomale experiments which demonstrated that an increase in *otu* and *Sxl* expression together, but not separately, causes a substantial improvement in germ cell differentia-

tion and in the frequency of nuclear SXL localization (Table 1). These pseudomale results are analogous to those obtained from genetic epistasis studies between certain *otu* and *Sxl* alleles, which were the first indications of functional or regulatory interactions between these two genes (Bae *et al.*, 1994; Pauli *et al.*, 1993). The semiconstitutive *Sxl* alleles could suppress the phenotype of partially functional *otu* allele combinations, but had no effect on the most severe *otu* mutations. We interpret these findings as indicating that in X/X germ cells arrested either by *otu* mutations or somatic sexual transformation, increased levels of SXL protein can support further oogenic development only if sufficient *otu* activity is present. Therefore, an *otu*-dependent process is required to establish conditions conducive for the optimal localization and function of the germline *Sxl* product.

How the dynamic redistribution of SXL during oogenesis relates to its function in the germline is unknown. In somatic cells, *Sxl* acts in the nucleus to regulate sex-specific RNA processing, including that of its own transcript (reviewed in Cline & Meyer, 1996). A similar autoregulatory mechanism has been suggested to occur in the germline (Bopp *et al.*, 1993; Hager & Cline, 1997), in which case a block in nuclear SXL localization by *otu* mutations should result in an increase in the male-specific splice form and a concomitant decrease in SXL protein levels. Such a phenotype has been reported for certain hypomorphic *otu* alleles (Bopp *et al.*, 1993; Oliver *et al.*, 1993; Pauli *et al.*, 1993). Therefore, the shuttling of SXL between the nucleus and cytoplasm may be a critical means of regulating early oogenic stages.

An unexpected finding was that SXL protein associates with one or more discrete sites on the chromosome in a pattern that appears to change as the egg chamber matures. We did not observe a similar localization on somatic

polytene chromosomes derived from salivary glands (data not shown), suggesting that this phenotype is germline-specific. How these SXL-associated chromosome sites might relate to the known functions of *Sxl* as a regulator of both RNA splicing and transcriptional efficiency is unclear. Furthermore, because the germline polytene chromosomes are produced in an *otu* mutant background, it is not known whether the same binding patterns occur in wild-type germ cells. Nevertheless, the fact that SXL can associate with chromosomes in a dynamic and developmentally specific manner provides circumstantial evidence that the nuclear localization is critical for *Sxl* germline function.

In addition to SXL protein, *otu* function is also required for the correct localization of several RNAs, including its own transcripts, during later stages of oogenesis (Tirronen *et al.*, 1995). In particular, it was noted that *oskar* RNA was dependent on wild-type levels of *otu* for its translocation to the posterior pole of the oocyte, a process requiring microtubules (Pokrywka & Stephenson, 1995; Theurkauf *et al.*, 1993). These observations are interesting, given other indications for an association between *otu* and cytoskeletal elements. For example, *otu* mutations affect the distribution of actin filaments during the maturation of fusomes and ring canals, and the formation of an actin cytoskeletal network necessary to anchor the nucleus of stage 10 nurse cells (Rodesch *et al.*, 1997; Storto & King, 1988). It may be that the intracellular localization of SXL involves some aspect of the germline cytoskeleton specifically controlled by *otu*.

Experimental procedures

Fly strains

Flies were raised on standard cornmeal, molasses, yeast and agar media containing propionic acid as a mould inhibitor and supplemented with live yeast. Unless otherwise noted, alleles and chromosomes used are as previously described (Lindsley & Zimm, 1992). The *hs-otu* construct places *otu* function under the control of the *Drosophila hsp70* promoter and has previously been described (Nagoshi *et al.*, 1995). The *ry*⁺ transgene is inserted into a third chromosome that is *ry* and *e*.

otu^{P^{Δ1}} 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^{Δ3}} and *otu*^{P^{Δ5}} 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 & Riley, 1982; Storto & King, 1988).

Immunohistochemistry

Adults were aged 4–7 days after eclosion unless otherwise noted. Gonads were dissected in PBS (130 mM NaCl, 7 mM Na₂HPO₄–2H₂O, 3 mM NaH₂PO₄–2H₂O). 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 four times in

PBT (0.1% Triton X-100, 0.05% Tween 80 in PBS) for 15 min, 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 overnight at 4 °C. Primaries included the following monoclonals: anti- α -spectrin (3A9, 1 : 200 dilution; from the laboratory of D. Branton), anti-SXL (1 : 50 dilution; American Type Culture Collection and University of Iowa Developmental Studies Hybridoma Bank), and a polyclonal VASA antibody preparation (1 : 1000) from the laboratory of L. & Y. Jan. Primaries were removed with three 15-min 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). When necessary, nuclei were labelled with propidium iodide. In this case, 125 μ g/ml RNase (Boehringer Mannheim) was added to the secondary antibody incubation solution. Preparations were mounted in Vectashield containing 1.5 μ g/ml propidium iodide (Vector Laboratories).

STAU-specific antibodies were a gift from the laboratory of D. St. Johnston (1 : 200). Secondary antibodies used were alkaline phosphatase-conjugated anti-mouse IgG (1 : 200; Molecular Probes). Peroxidase activity was detected using a diaminobenzidine-based detection kit (Sigma). The preparation was mounted in 50% glycerol/PBS.

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 LX450A camera for image capture. Figures were produced by transferring the captured images to Adobe Photoshop. All processing was 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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