**stall**-Mediated Extrinsic Control of Ovarian Follicle Formation in Drosophila

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ABSTRACT

Complex patterns of morphogenesis require intricate coordination of multiple, regulatory processes that control cellular identities, shapes, and behaviors, both locally and over vast distances in the developing organism or tissue. Studying Drosophila oogenesis as a model for tissue morphogenesis, we have discovered extrar ovarian regulation of follicle formation. Clonal analysis and ovary transplantation have demonstrated that long-range control of follicle individualization requires *stall* gene function in cells outside of the ovary. Although tissue nonautonomous regulation has been shown to govern follicle maturation and survival, this is the first report of an extraovarian pathway involved in normal follicle formation.

FOLLCLE formation in *Drosophila melanogaster* takes place in the germarium, which is located at the anterior tip of each ovariole (Figure 1A; for a review, see Spradling 1993). Both germline stem cells (GSC) and somatic stem cells (SSC) reside in the germarium and receive cues from the anteriorly positioned terminal filament cells for their division and maintenance. A germline cyst is initiated when a GSC divides asymmetrically to produce a single cystoblast, while regenerating the GSC. The cystoblast undergoes four rounds of mitosis with incomplete cytokinesis to form a germline cyst, composed of 16 interconnected cystocytes. One of these cystocytes becomes specified as an oocyte, while the remaining cystocytes become nurse cells. As the cyst matures and moves posteriorly through the germarium, somatic cells produced by SSC divisions move to surround each cyst to form a single-layered cuboidal epithelium. The completed follicle consists of a single germline cyst enveloped by the somatic epithelium and, as it exits the germarium, a single column of 6–10 somatic cells separates it from neighboring follicles for continued maturation.

The complex array of cellular events that contribute to ovarian follicle formation is regulated by an equally complex assortment of regulatory mechanisms. Signal transduction cascades that act within the ovary during the earliest stages of follicle formation include the *decapentaplegic* and *fs(1)Yb/piwi/hedgehog* pathways for the maintenance of GSC and SSC fates, as well as for the regulation of mitotic divisions (Xie and Spradling 1998; King and Lin 1999; Cox et al. 2000; King et al. 2001). Interfollicular communication is mediated by the *Notch/Delta* and *JAK/STAT* pathways (Torres et al. 2003), and intrafollicular communication requires *daughterless* (Cummings and Cronmiller 1994) for the specification of cellular identities in the established follicle. Intracellular regulators have been shown to control the establishment and maintenance of the cystoblast fate (e.g., *bag of marbles* (Chen and McKearin 2003) and *benign gonial cell neoplasm* (Lavoie et al. 1999)) or to monitor the balance between germline and soma production (*daughterless*; Smith et al. 2002). Finally, extraovarian signaling via the insulin pathway regulates germline cyst production in response to the nutritional state of the fly (Drummond-Barbosa and Spradling 2001). We show here that long-range signaling regulates follicle formation as well and that *stall* (*stl*) function is an essential component of this morphogenetic control.

MATERIALS AND METHODS

**Drosophila stocks:** Flies were maintained on molasses-cornmeal-yeast medium at 25°C. Fly stocks used in this study are listed in Table 1.

**Genetic analysis of stl:** Originally isolated as *fs(2)A16* (Bakan 1973), the *stl*<sup>stl</sup> allele fails to complement all other known EMS-induced alleles of *stl* (Jones 1999). To aid in the interpretation of mutant phenotypes, we subjected several of the various *stl* mutant chromosomes to recombination to remove unrelated extraneous mutations in linked genes.

**Staining and analysis of ovarian tissue:** Fixed ovarian tissue was stained with DAPI as described previously (Cummings and Cronmiller 1994). Stained ovaries were visualized in all cases on Zeiss Axiohot/Axioskope microscopes, and images were captured in black and white by either Pixera (germline clones) or Olympus Magnafire (all other experiments) digital cameras. Images were false-colored for GFP, fluorescein, and rhodamine in Adobe Photoshop.

**Larval and pupal gonad preparation:** Gonads were dissected from either wandering third instar larvae or pharate adult pupae and fixed in 4% paraformaldehyde. Fixed gonads were stained with antibodies against Hts (1B1) and Vasa, as described previously (Smith and Cronmiller 2001).

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TABLE 1

Drosophila stocks used in this study

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Mosaic analysis of *stl*: For germline clone analysis, progeny from an hs-FLP w/+; FRT G13 stlF16 × w; FRT ovoF10/CyO cross were heat-shocked at 37ºC for 2 hr on each of 2 consecutive days. Progeny were allowed to mature at 25ºC until eclosion. At 3–5 days posteclosion, ovaries were dissected, fixed in 4% paraformaldehyde, and stained with DAPI.

To generate marked somatic clones of *stlF187* and *stlF1977*, w/w; FRT G13 stl/CyO virgin females were crossed to hs-FLP w; FRT G13 GFP males. Progeny were heat-shocked at 37ºC and, after eclosion, w/w; FRT G13 stl/FRT G13 GFP flies were aged 4–7 days before ovary dissection. Tissue was fixed in 4% paraformaldehyde and stained with DAPI.

Ovary transplantation: Transplantation of germarial tissue from donor females to male hosts was performed according to the protocol outlined in Lin and Spradling (1993) with the following modifications: Germaria were dissected in Shields and Sang M3 insect medium (Sigma, St. Louis) and held at room temperature for no more than 20 min before transfer to male host abdomens. Hosts were allowed to recover for 11 days before dissection and staining with DAPI.

RESULTS

The *stl* mutant phenotype: Our analysis of the *stl* mutant phenotype endorsed initial assessments of this gene’s essential role during oogenesis (Bakken 1973; Schüpbach and Wieschaus 1991). We found follicle formation in adults to be severely disrupted, even in newly eclosed females. For example, the interfollicular stalks that normally separate adjacent wild-type follicles were completely absent in *stl* ovarioles (Figure 1, B and C). In addition, these mutant ovarioles lacked the somatic epithelial layers that normally envelop individual germline cysts. As a consequence of these defects, each *stl* ovariole appeared essentially as a single irregular somatic epithelium that contained multiple germline cysts. This phenotype resulted from failed follicle individualization, rather than from persistent germline cell division, because mutant ovarioles contained germ cells at varying stages of maturation and because groups of 16 interconnected germ cells that would ordinarily have identified a single germline cyst were still recognizable. Moreover, this disruption of oogenesis in *stl* mutant adults was exacerbated by degeneration of both germline and somatic cells (Figure 1C). These ovary defects were identical not only in homozygotes of all five known alleles of *stl*, but also in all heteroallelic combinations of these alleles (Figure 1D, for example). We were unable to document unambiguously the *stl* hemizygous phenotype, since all of the putative deletion chromosomes that should have uncovered the *stl* gene's essential role during oogenesis (Figure 1C) endorsed initial assessments of this locus proved to be structurally complex. Nevertheless, because of the similar nature of the *stl* homozygous and heteroallelic follicular defects and because none of the *stl* alleles has ever been observed to have any dominant effects (data not shown), the ovarian phenotype associated with these alleles most likely represents the loss of *stl* gene function.

Since ovarian follicle formation was completely disrupted already in newly eclosed *stl* mutant females, we examined larval (third instar) and pupal gonads to determine the onset of the *stl* phenotype. On the basis of the organization of germline and somatic cells, larval gonads looked morphologically normal (Figure 2, compare A with B). Pupal gonads, however, lacked complete follicle individualization: Although adjacent germline cysts were mostly separated from each other by clusters/layers of somatic cells, there was no evidence of interfollicular stalks (Figure 2, compare C with D). Thus, wild-type *stl* function must be required at least as early as the pupal stage for normal follicle morphogenesis. To address the question of whether *stl* normally functions in the germline and/or somatic cells of the ovary, we carried out clonal analysis.

Mosaic analysis of *stl*: Clonal analysis of *stl* function revealed unexpected and complex cellular requirements for the gene, failing to detect exclusive requirements for the gene in either the germline or the soma.
We first used the dominant female sterile technique to generate stl+ germline clones that were characterized by the absence of the ovoD1 phenotype (DFS-FLP/FRT; CHOU and PERRIMON 1996). Since ovoD1 homozygous follicles arrest at stage 4, germline clones were recognizable as follicles that were more mature. We induced mitotic recombination during larval stages and recovered ovo+ clones in 19% of ovarioles (n = 279; Figure 3A). Of 54 stl+ clones, only 7 showed weak stl-like follicle formation defects (Figure 3B). Among the remaining 225 stl+ (ovoD1) ovarioles, 41 showed similar follicle formation defects, superimposed on the characteristic ovoD1 phenotype (Figure 3C). The frequency of defects in ovarioles with mutant germline clones (13%) was not statistically different from that in ovarioles without (18%; Student’s t-test, P = 0.67), suggesting that stl function is not required in the germline for normal follicle morphogenesis. To determine whether the follicle defects observed in this experiment resulted from the perturbation of stl function in critical somatic cells, unrelated to the stl genotype in the germline, we generated stl mutant somatic clones that were identifiable by the absence of a GFP marker (LUSCHNIG et al. 2000). Marked mutant clones were recovered in 14–84% of ovarioles, depending on the recombination induction protocol used; two independent stl alleles produced essentially indistinguishable results. Mutant clones were found in all somatic cell types, including the follicular epithelium, as well as the terminal filament (TF), cap (CAP), and inner sheath (IS) cells of the germarium. Clones restricted to the small population of specialized somatic cells in the germarium (TF, CAP, and IS) were not associated with any increase in the frequency of defective follicles (data not shown); however, we found that stl mutant clones in somatic epithelial cells were variably associated with follicular defects. To identify clone features that were most likely to result in follicular defects, we analyzed the stl somatic clones in greater detail.

Severe stl-like follicle formation defects were most often associated with large stl clones in the somatic ovary; however, not every large somatic clone exhibited such severe defects. We classified follicular defects as severe, weak, or absent, while simultaneously categorizing the corresponding ovarioles according to whether they contained large, small, or no somatic clones. Severe morphological defects were found exclusively in ovarioles that contained mutant clones. Further, gross follicular disruptions were most often correlated with large clones that encompassed a majority of the somatic epithelium (Figure 4A), although they occasionally coincided with small mutant patches (Figure 4B). Phenotypic severity, however, was not strictly correlated with somatic clone size, since rare large clones were recovered that exhibited no visible defects (Figure 4C). Similarly, while weak follicular defects were often associated with small clones, they were just as often present in ovarioles that lacked clones altogether (Figure 4D).
female percentage of defects in the controls was 7.9%; only two females exhibited percentages >5% (Figure 4E). In contrast, among experimental females the percentage of defective ovarioles per female was mostly >5% and reached as high as 30%. In addition to this clustering of ovarian defects, an increased incidence (contingency \( \chi^2, P < 0.1 \)) of weak defects in experimental (6.0%) vs. control (2.9%) females suggests that weak defects often represent the impact of nonovarian \( stl \) clones. Overall, these mitotic clone data suggest a model in which \( stl \) normally contributes to ovarian follicle formation not only in somatic cells of the ovary itself, but also through a previously unidentified regulatory pathway that originates in cells outside of the ovary. Moreover, the extraovarian cells that express \( stl \)'s function are probably no longer dividing in adults, since we did not recover significant ovarian defects (0.8% of ovarioles) when clones were initiated in adults, whereas \( \sim 11\% \) of ovarioles had defects when mutant clones were induced during larval stages. We tested our nonautonomy model by performing ovary transplantations.

**Ovarian transplantation analysis:** We carried out reciprocal ovary transplantations between genetically wild-type and homozygous \( stl \) mutant adults and confirmed an extrinsic, i.e., extraovarian, role for \( stl \) during ovarian follicle formation. Wild-type control transplants confirmed that oogenesis proceeded normally following transfer of germlarial tissue to the abdomens of male hosts (Figure 5A; Lin and Spradling 1993). Transplantation of wild-type germaria into two different \( stl \) host genotypes, however, resulted in morphologically abnormal ovarioles that were similar to, but not as severe as, those of homozygous mutant females. In some cases, recovered ovarioles did contain portions of interfollicular somatic epithelium, which has never been observed in \( stl \) homozygous ovaries (Figure 5B). However, all cases showed one or more typical \( stl \) ovarian defects, including germline cyst mispackaging, absence of interfollicular stalks, and widespread cellular degeneration (Figure 5B and C). Reciprocal transplantations, namely \( stl \) mutant ovary tissue into wild-type hosts, were problematic, since transplanted tissue was never recovered in these experiments. However, the number of transplants performed and the host survival rates were comparable in both transplant operations, suggesting that the \( stl \) mutant tissue may have been too fragile to survive the stringent transplantation procedure. Although our transplant experiments could not address the sufficiency of extraovarian \( stl \) function for normal oogenesis, they do directly demonstrate the necessity for such a function.

**DISCUSSION**

Our results collectively support a model in which \( stl \) function is essential for proper ovarian follicle formation both in somatic cells in the ovary and in extraovarian somatic cells. Indeed, although hormone regulation...
Figure 3.—Clonal analysis of stl in the germline. Clones were initiated in first and second instar larvae; germline mosaics were identified by the loss of the ovoD1 phenotype. (A) Despite the stl clone in the germline, an ovo+/stlH11001 ovariole forms normally maturing, discrete follicles (e.g., large arrow). An ovoD1/stl ovariole in the same ovary (small arrow) shows the absence of maturing follicles that is typical of the ovoD1 mutant phenotype. (B) Follicular defects were observed occasionally in ovo+/stlH11001 ovarioles. In this example more than one germline cyst is enveloped by a single somatic epithelium (arrowhead). (C) Similar follicular defects (arrowheads) are shown in ovoD1/stlH11001 ovarioles. All ovaries were stained with DAPI to visualize tissue organization. Bars, 100 μm.

has been linked to a germline survival checkpoint in flies (Drummond-Barbosa and Spradling 2001), our discovery of stl’s extraovarian contribution to oogenesis provides the first evidence for a tissue nonautonomous pathway that regulates follicle individualization. While our transplantation experiments definitively demonstrate the requirement for stl function outside of the ovary, our interpretation that the gene is simultaneously required within the ovary is supported by several observations. First, our inability to recover stl tissue from wild-type transplant hosts could indicate an essential stl ovarian function, especially if that function were required prior to the patterning of the adult ovariole during pupal gonadogenesis, since stl mutants already show follicle defects in pupal ovaries. However, an alternative interpretation could be that the transplanted stl tissue simply did not survive our transplantation procedure. More significantly, the phenotypic defects exhibited by the wild-type ovarian tissue grown in stl mutant hosts were less severe than those associated with the ovaries of homozygous mutant females. Finally, in our clone generation experiment, weak defects were observed even in the absence of ovarian clones, and severe defects were most often observed when large ovarian clones were present. We propose that weak follicular defects arising from clones outside of the ovary were exacerbated by the simultaneous disruption of stl function within the ovarian soma. Thus, we conclude that both the somatic ovarian and the extraovarian roles for stl are essential for proper folliculogenesis, but neither is sufficient, indicating that these functions are nonredundant.

Our evidence for stl’s extraovarian participation in ovarian follicle formation raises a number of intriguing questions about the nature of such a regulatory process. Since stl males appear morphologically normal and yet perturbing stl function in males (homozygous stl) was enough to disrupt oogenesis in transplanted ovarioles, the stl-mediated signaling pathway could be functionally female specific under normal conditions, but inducible in males by transplanted ovarian tissue. Alternatively, it is possible that stl contributes to a universal signal that is normally present in both males and females. In this case, at least one of its primary targets would be ovary specific and thereby female specific. Of the five extant alleles of stl, four were recovered from an exclusively female sterile mutagenesis screen (Schüpbach and Wieschaus 1991), while the remaining allele was isolated in a nonsaturating sex nonspecific sterility screen (Bakken 1973). It is possible, therefore, that any or all of these alleles of stl are female-specific lesions in a gene with broader functions. Regardless of the specific-
ity of the signal’s origin, no particular cellular mechanism during follicle formation that requires the long-range signal has yet been discovered; however, it has been shown that stl is not required for expression of the polar or interfollicular stalk cell fates in the soma (Tworoger et al. 1999). Molecular characterization of stl, together with genetic interaction studies, should provide clues to the identities of ovarian targets of the long-range signaling, defined by this gene’s function. One intriguing possibility is the transcriptional regulatory gene, daughterless, on the basis of its strong dominant mutant interaction with stl (Smith and Cronmiller 2001). Finally, since hormones are known to control checkpoints in oogenesis both early (Drummond-Barbosa and Spradling 2001) and late (Soller et al. 1999), it is possible that stl is directly involved in generating or sending a similar hormonal signal to control follicle formation. Such a signal could originate in the nervous system, given the numerous peptides and hormones that are synthesized in specialized cells in the brain and consistent with the paucity of ovarian defects observed following clone induction in adults.

The discovery of extraovarian control of Drosophila follicle formation prompts the question of whether similar long-range signaling is involved in mammalian folliculogenesis. There are certainly similarities between Drosophila and mammals with respect to follicle maturation and survival, given that those stages of oogenesis in both systems are regulated indirectly via the alteration of ecdysone levels (in response to juvenile hormone) (Jowett and Postlethwait 1980; Soller et al. 1999) and progesterone synthesis (in response to follicle stimulating hormone) (Chun et al. 1996; Makrigiannakis et al. 2000), respectively. Another striking similarity is the importance of intraovarian regulation that mediates extensive cell-cell communication between the oocyte/germline and the surrounding somatic cells in control of cell proliferation or tissue organization; such regulation involves a number of signal transduction pathways, including Notch or EGFR (in Drosophila) (Goode et al. 1992; Torres et al. 2003) and GDF-9 or bFGF (in mammals) (Fortune 2003). Little is known, however, about the control of mammalian oogenesis prior to follicle activation and growth, and it is the early events, including the migration of granulosa cells to encapsulate individual oocytes, that are most analogous to Drosophila follicle formation, during which somatic cells migrate to surround individual germline cysts. Finally, Drosophila and human follicle formation share the same practical restrictions with respect to in vitro culturing. Only Drosophila follicles that are fully formed at the time of explant complete maturation during ovary in vitro culturing; this experimental limitation has hindered our progress toward understanding how follicle formation works at the cellular level. Similarly, cultured preantral human follicles exhibit low meiotic competence, uncoordinated granulosa cell and oocyte growth, and failed

Figure 4.—Variation in phenotypic severity among marked stl mutant clones. stl mutant clones in an otherwise wild-type background were initiated in first and second instar larvae and identified by the absence of a GFP marker (A, B, C, and D); DAPI staining reveals the structure of these ovarioles (A’, B’, C’, and D’). (A and A’) A large somatic clone that encompasses an entire ovariole (GFP-stl is outlined) exhibits follicle formation defects that are as severe as those observed in homozygous stl ovaries. (B and B’) An ovariole with relatively small patches of stl mutant soma (outlined) shows severe follicular defects. All other somatic cells, including TF, IS, and CAP, are wild type in this ovariole. (C and C’) In another ovariole, however, follicles are formed properly, in spite of a large area of stl mutant soma (outlined). (D and D’) Weak follicular defects (arrowhead) are present in an ovariole that contains no stl mutant clones. Bars, 100 μm. (E) Increased incidence of multiple follicular defects among mutant (FRT GFP/FRT stl or FRT GFP/FRT stl(FRT) vs. wild-type (control, FRT GFP/FRT stl) females, following clone induction. Each bar represents a single female; the percentage of follicle defects is indicated above each bar. Follicular defects were scored independently of clone frequencies.
maturation (Smittz and Cortvrindt 2002); the inability to culture these early stage follicles has eliminated them as a source of oocytes for alternative reproductive strategies. It is an exciting possibility that preindividualized Drosophila follicle stages and preantral human follicles represent comparable premeiotic stages of oogenesis. If they involve comparable extrinsic physiologic controls, the stall-mediated signaling function could identify a more universal regulatory mechanism; it could be the missing ingredient that is needed for successful in vitro culturing of both tissues. The molecular identification of stall will allow this possibility to be explored further.

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