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Evolution of Developmental Control Mechanisms

The roles of cell size and cell number in determining ovariole number in *Drosophila*

Didem Pelin Sarikaya [a], Abel A. Belay [a], Abha Ahuja [a], Aisha Dorta [b], Delbert André Green II [c], Cassandra G. Extavour [a,*]

[a] Department of Organismic and Evolutionary Biology, Harvard University, 16 Divinity Avenue, Cambridge, MA 02138, USA
[b] Brooklyn College, City University New York, 2900 Bedford Avenue, Brooklyn, NY 11210, USA
[c] Department of Molecular and Cellular Biology, Harvard University, 16 Divinity Avenue, Cambridge, MA 02138, USA

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ABSTRACT

All insect ovaries are composed of functional units called ovarioles, which contain sequentially developing egg chambers. The number of ovarioles varies between and within species. Ovariole number is an important determinant of fecundity and thus affects individual fitness. Although *Drosophila* oogenesis has been intensively studied, the genetic and cellular basis for determination of ovariole number remains unknown. Ovariole formation begins during larval development with the morphogenesis of terminal filament cells (TFCs) into stacks called terminal filaments (TFs). We induced changes in ovariole number in *Drosophila melanogaster* by genetically altering cell size and cell number in the TFC population, and analyzed TF morphogenesis in these ovaries to understand the cellular basis for the changes in ovariole number. Increasing TFC size contributed to higher ovariole number by increasing TF number. Similarly, increasing total TFC number led to higher ovariole number via an increase in TF number. By analyzing ovarian morphogenesis in another *Drosophila* species we showed that TFC number regulation is a target of evolutionary change that affects ovariole number. In contrast, temperature-dependent plasticity in ovariole number was due to changes in cell–cell sorting during TF morphogenesis, rather than changes in cell size or cell number. We have thus identified two distinct developmental processes that regulate ovariole number: establishment of total TFC number, and TFC sorting during TF morphogenesis. Our data suggest that the genetic changes underlying species-specific ovariole number may alter the total number of TFCs available to contribute to TF formation. This work provides for the first time specific and quantitative developmental tools to investigate the evolution of a highly conserved reproductive structure.

Introduction

All insect ovaries are composed of highly conserved functional units called ovarioles (Büning, 1994). Ovariole number varies within and between species (Büning, 1998; Markow and O'Grady, 2007; Telonis-Scott et al., 2005). Because each ovariole produces eggs autonomously (Extavour and García-Bellido, 2001; R' kha et al., 1997), the number of ovarioles is an important determinant of fecundity (Cohet and David, 1978; David, 1970; R' kha et al., 1997), thereby influencing evolutionary fitness (Orr, 2009). It is therefore important to understand the developmental mechanisms that regulate ovariole number. This will inform our understanding of how evolutionary changes in these mechanisms might lead to ovariole number differences, and thus fitness differences, within and between species.

Ovariole development and function are best understood in *Drosophila melanogaster*. Each ovariole consists of an anterior germarium and maturing egg chambers, or follicles. The germarium houses germ line stem cells that divide to produce oocytes (Wieschaus and Szabad, 1979). As follicles leave the germarium, they move posteriorly and continue to develop to form mature oocytes. *D. melanogaster* ovaries consist of approximately 16 to 23 ovarioles (depending on the strain). Ovariole number is determined during larval development through the morphogenesis of somatic structures called terminal filaments (TFs), each of which is composed of a stack of seven to ten terminal filament cells (TFCs) (Godt and Laski, 1995; King et al., 1968). TFC specification begins at the second larval instar (L2; Fig. 1A), and proceeds until the onset of the pupal stage (LP; Fig. 1D) (Godt and Laski, 1995; Sahut-Barnola et al., 1995). TFs form during the late third larval instar (L3; Fig. 1B, C) by intercalation of TFCs in a medial to lateral progression across the ovary (Godt and Laski, 1995). As TF formation is completed, apical somatic cells migrate posteriorly between the TFs, secreting a basement membrane that separates TFs from each other. The progressive posterior migration of these apical cells encapsulates two to three germ line stem cells, and several early oogonia, into each forming ovariole. Finally, a stack of basal stalk cells is incorporated into the posterior end of each ovariole. These stalk cells ultimately connect ovarioles to the oviduct, providing an outlet for the oocytes formed in each ovariole (King, 1970; King et al., 1968). Because TFs serve as beginning points for ovariole formation, elucidating how TF number is established is

* Corresponding author. Fax: +1 617 496 9507.
E-mail address: extavour@oeb.harvard.edu (C.G. Extavour).

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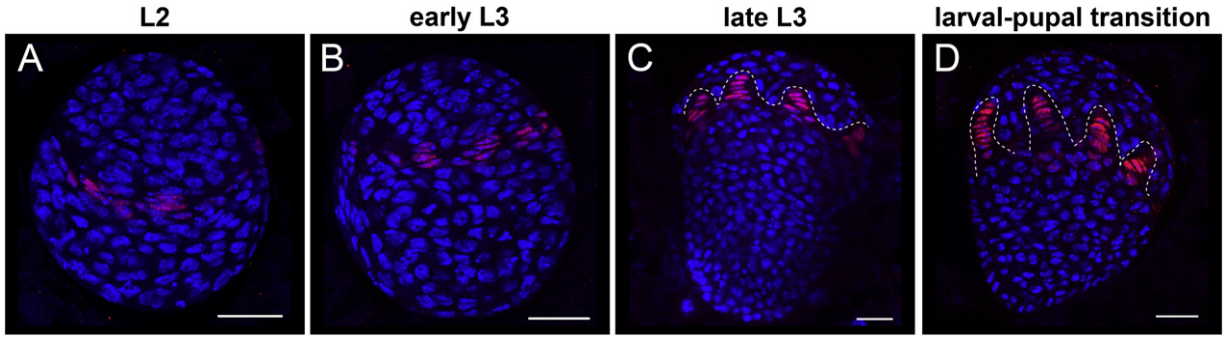

Fig. 1. Terminal filament cell (TFC) specification, and TF morphogenesis during larval development in *D. melanogaster*. Progressive specification and intercalation of TFCs (red) begins in the second larval instar L2 (A) and progresses throughout the third larval instar L3 (B, C). Mature terminal filaments (TFs) are found at the larval–pupal stage (D). Dotted line in (C, D) outlines the forming TFs. Red: Engrailed; blue: Hoechst. Anterior is up;. Scale bar = 20 μm.

critical to understanding the developmental and evolutionary basis of ovariole number.

Because TFs are neither created nor destroyed during normal pupal development in *Drosophila* (King, 1970), TF number at the larval–pupal transition determines adult ovariole number (Hodin and Riddiford, 2000). Ovarioles can form in the absence of germ cells (Aboïm, 1945; Engstrom et al., 1982), and changes in germ cell number do not induce changes in TF number (Barnes et al., 2006; Gilboa and Lehmann, 2006). The germ cell population thus does not have a major influence on ovariole number. This suggests that developmental processes that form and sort the somatic cells that create TFs, the TFCs, determine changes in ovariole number.

Although *D. melanogaster* oogenesis has been intensively studied, the formation of ovarioles during ovarian morphogenesis is still not well understood. Specifically, the genetic and cellular basis for determination of ovariole number remains unknown. Correct regulation of size and number in other organs, including wings in flies and somites in frogs (Cooke, 1975; Resino and Garcia-Bellido, 2004), relies on the coordination of cell number (proliferation), cell size (growth), and cell sorting behavior. Moreover, evolutionary change in body size is thought to be the result of changes in the numbers and sizes of cells (French et al., 1998; James et al., 1995; Partridge et al., 1999). We therefore hypothesized that the developmental parameters influencing ovariole number might include the numbers, sizes, and cell sorting behaviors of TFCs. In this context, we analyzed TFC number, size and morphogenesis in ovaries with genetically- or environmentally-induced differences in ovariole number. To assess the role of TFC size in determining ovariole number, we changed the activity of S6 kinase (S6K), which is a downstream regulator of Insulin/TOR signaling (reviewed by Fenton and Gout, 2011b). Altering S6K activity changes cell size without affecting cell number in ectodermal tissues (Montagne et al., 1999). We also assessed the role of TFC number in regulating ovariole number, by manipulating the activity of the Hippo pathway. This recently described pathway plays a conserved role in controlling cell number in fruit flies and mammals, but does not alter cell size (Dong et al., 2007; Harvey et al., 2003; Wu et al., 2003). Based on the data from these manipulations, we propose a model for the major developmental processes that regulate changes in ovariole number.

We used this model to investigate the developmental basis of evolutionary change in this trait. Ovariole number is species-specific and largely genetically determined. Intra- and inter-species genetic studies on ovariole number indicate that genetic variation in the trait is additive and polygenic (Coyne et al., 1991; Orgogozo et al., 2006; Telonis-Scott et al., 2005; Wayne and McIntyre, 2002; Wayne et al., 2001). To determine the roles of TFC size, number, and sorting behavior in evolutionary change in ovariole number, we compared TF morphogenesis in two *Drosophila* species with different ovariole numbers. Finally, we addressed the role of these cell biological parameters in phenotypic plasticity in ovariole number. Environmental inputs such as temperature and nutrition can also influence adult ovariole number (Bergland et al., 2008; Hodin and Riddiford, 2000). To assess the reasons for ovariole number changes induced by rearing environment, we compared (1) flies reared at two different temperatures, and (2) flies reared on standard or reduced nutrition, and analyzed TFC behavior. Our data suggest that genetic and environmental variation can affect ovariole number through different developmental processes.

Materials and methods

Fly strains

TRiP (Harvard Medical School) RNAi lines used to knock down Hippo pathway members were y^1v^1; *P{TRiP^{hpo}}attP2* (Bloomington *Drosophila* stock center 33614; abbreviated to *UAS:RNAi^{hpo}*) and y^1v^1; *P{TRiP^{wts}}attP2* (Bloomington *Drosophila* stock center 27662; abbreviated to *UAS:RNAi^{wts}*). These lines were selected as they have been reported to increase cell proliferation in the gut epithelium of flies (Karpowicz et al., 2010). Mutant S6K allele lines used were *w; P{w^{+mC}=UAS-S6k.TE}2* (Bloomington *Drosophila* Stock Center 6912) and *w; P{w^{+mC}=UAS-S6k.STDE}2/CyO actinGFP*, (derived from Bloomington *Drosophila* Stock Center 6913 and 4533; abbreviated to *UAS:S6K^X*). These lines were selected as they have been reported to increase cell size (but not cell proliferation) in the wing (Barcelo and Stewart, 2002). The GAL4 driver lines used were *w; P{GawB}bab1$^{Pgal4-2}$/TM6, Tb^1* (Bloomington *Drosophila* Stock Center 6803) (Cabrera et al., 2002) and *nubbin:GAL4* (gift of Tassos Pavlopoulos), abbreviated to *bab:GAL4* and *nub:GAL4*, respectively. The *bab:GAL4* driver is expressed in somatic cells of the larval ovary, most strongly in the somatic cells anterior to the germ cells, which are largely destined to become TF cells (Cabrera et al., 2002). Additional somatic cell populations expressing this driver at lower levels are the intermingled cells in direct contact with germ cells, and at late L3 and prepupal stages, the somatic cells posterior to the germ cells; neither of these latter cell populations contributes to terminal filaments. The *bab:GAL4* driver is not expressed in germ cells. GAL4 line virgins were crossed to *UAS:RNAi^{hpo}*, *UAS:RNAi^{wts}*, *UAS:dS6K^{TE}* and *UAS:dS6K^{STDE}* males. *Drosophila yakuba* (UC San Diego *Drosophila* Stock Center 1402–0261.01 via Daniel Hartl's lab) was maintained at 25 °C for all experiments.

Rearing conditions: variation of temperature and nutritional regimes

Temperature sensitive experiments were conducted with OregonR-C flies (Bloomington *Drosophila* Stock Center 5 via Daniel Hartl's lab). Flies were reared at 25 °C or 18 °C at 60% humidity on standard fly medium (0.8% agar, 2.75% yeast, 5.2% corn meal, 11% dextrose) for at least two generations before experiments were conducted (Fig. S1A). Because reduced nutritional intake of larvae resulting from crowded tubes can reduce adult ovariole number, adults were permitted to lay eggs in vials for two to six hours and then removed from the vial to prevent overcrowding of larvae. Only tubes containing fewer than 100 pupae were