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Evolution of Developmental Control Mechanisms

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

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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

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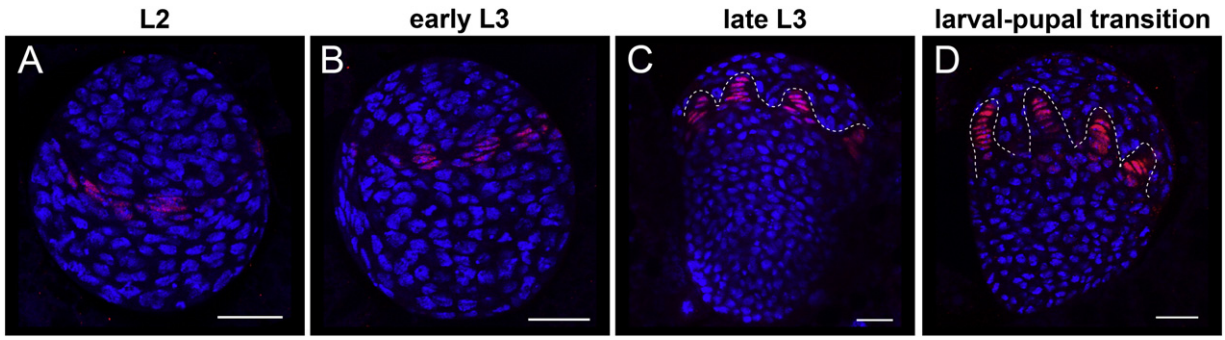

Fig. 1. Terminal filament cell (TFC) specification, and TF morphogenesis during larval development in *D. melanogaster*. Progressive specification and intercalation of TFCs (red) begins in the second larval instar L2 (A) and progresses throughout the third larval instar L3 (B, C). Mature terminal filaments (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

used for analysis of larval–pupal ovaries, and for counts of ovariole number in adults. Adults hatched from these tubes were used to create new parent cultures at the same temperature. For starvation experiments, flies were reared at 25 °C on 1/4 standard fly medium ("quarter food") made by mixing one part standard fly medium with three parts 3% agar (VWR); overcrowding of larvae was prevented as described above.

Adult analysis: ovariole number

As described above, only tubes containing fewer than 100 larvae were used for all experiments. Adult female flies from non-crowded tubes were placed in 70% ethanol until sedated, and ovaries were dissected in 1× PBS/0.01% Triton X-100. Ovariole number was counted in 1× PBS under a dissecting microscope using tungsten needles. At least 20 ovaries were analyzed for each strain. For temperature comparisons, a two-tailed *t*-test was conducted using Microsoft Excel. Ovariole number comparison for Hippo pathway and S6K experimental adults were conducted by one-way analysis of variance (ANOVA), followed by Tukey's Honestly Significant Difference (HSD) using JMP (SAS Institute Inc., Cary, NC). ANOVA is a standard statistical method based on Fisher's methodology (Fisher, 1918) for determining whether significant differences exist between means from multiple groups; the ANOVA F statistic is the ratio of the variance of the means of different groups, to the variance between samples comprising a group, and is reported in the relevant figure legends for all data. The HSD test is a method based on pairwise comparisons of means in order to determine which means are significantly different from each other (Bremer and Doerge, 2010).

Adult analysis: wing cell size and number

Rearing temperature was reported to affect wing cell size but not number (Azevedo et al., 2002). We confirmed these results in our experimental conditions by analyzing wing cell size and density in flies used in our experiments. Wings were removed from adults and placed in 100% ethanol overnight. Wings were then washed in 70% ethanol, 1:1 ethanol:glycerol, and 50% glycerol in distilled water for 10 min, and mounted in 50% glycerol. Mounted wings were imaged using a Zeiss AxioImager Z1 and a Zeiss MRm AxioCam driven by AxioVision v4.6, and total surface area of the wing, cell number per area of interest, and cell size were measured using AxioVision v4.6 or Adobe Photoshop CS3. Total surface area was measured for the entire wing. Cell number per unit area was measured in the ventral region of compartment C of the wing (Baena-Lopez et al., 2005); the number of bristles was counted for the same surface area region of interest in different wings. Cell size was measured by selecting a single bristle, and connecting the surrounding six bristles to obtain the surface area. Alternatively, the distance between trichomes was measured and taken as the diameter of the cell to calculate cell area. Comparable cell size values were obtained with both methods. At least ten cells were measured per wing to obtain the individual's average wing cell size.

Immunohistochemistry

For larval analysis, the transition stage between the larval and pupal stages was used (referred to as "larval–pupal stage" throughout). Pupae with hardened, white pupal cases (Ashburner et al., 2005) were collected from vials containing less than 100 pupae. This stage was chosen for analysis because TF formation, which is gradual throughout the third larval instar, ends at the larval–pupal stage, and so the TF number of these ovaries is the final TF number for that individual. Ovaries with incomplete TFs (still in the process of intercalating) were discarded from the dataset, and only ovaries where all TFs were separated by migrating anterior cells were used. Samples were dissected in 1× PBS, fixed in 4% paraformaldehyde/1× PBS for 25 min at room temperature, and blocked in 0.5% goat serum (Jackson ImmunoLabs) in 1X PBS/0.01% Triton-X for 30 min at room temperature. Primary antibody incubation in mouse anti-Engrailed (Developmental Studies Hybridoma Bank 4D9, 1:40) and/or guinea pig anti-Traffic jam (gift of D. Godt, 1:4000) in blocking solution was conducted overnight at 4 °C. Engrailed labels the TF population (Forbes et al., 1996), and Traffic jam (Tj) labels intermingled cells and cap cells (Li et al., 2003). Samples were washed in 1X PBS/0.01% Triton-X twice for 15 min at room temperature, and incubated with FITC-Phalloidin or A555-Phalloidin (Invitrogen, 1:120 of 200 U/ml stock solution), Hoechst 33342 (Sigma, 1:500 of 10 mg/ml stock solution), and goat anti-Mouse Alexa 568 (Invitrogen, 1:500) and/or goat anti-Guinea Pig Cy5 (Jackson ImmunoLabs, 1:500) overnight at 4 °C. Samples were mounted in Vectashield mounting medium (Vector labs), and imaged using a Zeiss LSM 710 confocal microscope.

Larval analysis: TFC number per TF

Z-stack confocal images of stained ovaries were taken with a 40× objective and 1.2–1.6× zoom to capture the entire ovary at 1 μm intervals (Fig. S1B). Total TF number was counted and comparisons between samples were conducted using a two-tailed *t*-test. TFCs were identified by morphology and Engrailed expression. Engrailed-expressing cuboidal cells at the posterior of the TF were excluded from the TFC number count, as they were adjacent to germ cells and had characteristics of cap cells. Ten ovaries were analyzed for each condition for the environmental manipulations, and five individual ovaries for each genetic condition were analyzed for TFC number per TF. The dataset for each manipulation (temperature/genetic) contained measurements of several cells from each of for five or ten individuals, which were randomly selected. To account for potential individual variation affecting the dataset, we conducted a nested mixed model ANOVA (JMP, SAS Institute Inc., Cary, NC) with a fixed manipulation term (genetic or environmental) and a random-effects individual term nested within manipulation. Sample sizes reported reflect the individual ovary number, rather than the number of measurements made per individual, unless indicated otherwise.

Larval analysis: TFC size

Larval TFC size (=volume in μm^3) was obtained specifically in the third and fourth TFC of each TF, counting from the anterior tip of the TF in order to ensure the cells were comparable in size (Fig. 2A). Cell outlines were visualized by Phalloidin staining. For each individual ovary, four to ten cells (average 7.8 cells per sample) were analyzed by measuring the surface area of the cell through serial confocal image stacks for all stacks where the selected cell was visible (Fig. 2A). The sum of the surface areas was multiplied by the thickness of each individual stack to obtain the cell size. In the case of GAL4/UAS experiments, the maternal strain (*w; babGAL4$^{p4.2}$/TM6b, Tb^{1}*) and F1 siblings (*w; UAS-S6K^{X}/+; TM6b/+*) carry the TM6b balancer that contains a mutant allele of the gene *Tubby*. Flies carrying this *Tubby* allele are visibly shorter and stouter than wild type as adults and larvae (Linsdsley and Zimm, 1992). The mechanistic causes for the phenotype are unknown, but they may affect cell size. Because cell size is one of the parameters under analysis, and the *TM6b* chromosome is not present in any experimental animals of interest, we excluded these genotypes from the analysis. Similar to TFC number per TF, a mixed model nested ANOVA (see above) was used to analyze the data by setting temperature/genetic treatment as a fixed effect, and the individual nested within temperature/genetic treatment as random effect using JMP (SAS Institute Inc., Cary, NC).

Larval analysis: total TFC number

Obtaining images of larval ovaries where all TFCs can be resolved at the late L3 and larval–pupal stage is inefficient. As an alternative, we estimated total TFC number by calculating an average TFC number per TF (by averaging measurements from more than eight TFs; Fig. 2B), and multiplying by the TF number of that ovary (Fig. 2C).

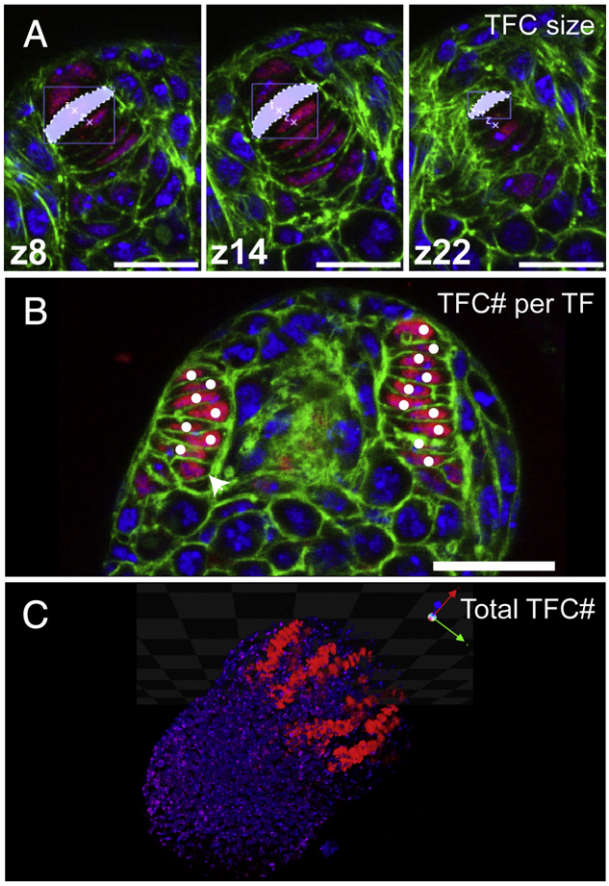

Fig. 2. Methodology for measuring TFC parameters in late larval ovaries. (A) Examples of optical sections used to measure TFC size. Measurements of TFC sizes were performed on the third and fourth cell from the anterior of the TF. For a given TF cell (identifiable by Engrailed-positive signal and flattened morphology), the surface area (visible with phalloidin-labeled cell outlines) is measured in every optical section where the cell is present, and cell size is obtained by multiplying the sum of the surface area measurements by the thickness of the optical sections. Examples of three Z-plane optical sections through a TF are shown. Optical section thickness is uniform for all images of a given ovary. (B) Example of an optical section used to count TFC number per TF. TFCs are identified (white dots) as engrailed-positive cells (red) with flattened nuclei in stacks. Cuboidal cells at the posterior of stacks with lower levels of Engrailed signal (arrowhead) are not included in TFC number counts, as they are cap cell precursors. (C) Examples of optical section reconstructions used to count total TF number. Sections are taken through a larval–pupal stage ovary, and reconstructed in three dimensions in order to visualize TFs where all TFCs are visible; the average number of TFCs per TF is then multiplied by the total TF number for that ovary (see Materials and methods and Fig. S1B). To count TF number manually (Fig. S1B), all optical sections of an ovary were examined. Red: Engrailed; blue: Hoechst; green: phalloidin. Anterior is up and scale bar = 6 μm in A and B; in C anterior is to the top right and scale bar = 10 μm.

We tested the validity of this method by randomly choosing eight TFC number per TF measurements from ovaries where total TFC number had also been counted manually, and comparing the calculated and counted measurements. For all eight ovaries analyzed, our calculation method gave total TFC numbers that matched the counted number with an accuracy of ±4 (average TFC number was 134.5 for manual counts and 135.8 for proxy calculation; $p = 0.79$, two-tailed t-test; Fig. S1D). Calculating total TFC number in this manner thus provides an accurate proxy for total TFC number.

Results

Constitutively active S6K in TFCs results in increased adult ovariole number

We hypothesized that TF number would be affected by cellular behaviors during TF morphogenesis. Specifically, we examined TFC size (Fig. 2A), TFC number per TF (Fig. 2B), and total TFC number (Fig. 2C) to gain insight into how the dynamics of TF morphogenesis could affect TF number, and therefore ovariole number. To test whether changes in TFC size could influence TF number, we used mutations in *S6 Kinase* (*S6K*) as a tool to change cell size. *S6K* phosphorylates the ribosomal subunit S6 and as a result, regulates translation downstream of the Insulin and TOR signaling pathways (Jefferies et al., 1997). Expression of constitutively active *S6K* alleles in the wing increases cell size, but does not alter cell number (Montagne et al., 1999) (Fig. S2). We took advantage of the GAL4/UAS system (Brand and Perrimon, 1993) to increase S6K activity in the TFC population (Fig. S3) with a *bab:GAL4* driver line (Cabrera et al., 2002) (see Materials and methods).

Expression of two different constitutively active alleles of *S6K* ($S6K^{TE}$ and $S6K^{STDE}$; see Materials and methods) in TFCs resulted in an increase in ovariole number of females from the experimental cross (Fig. 3A, B). Ovariole number in the experimental F1 flies (*w; UAS-S6K^{X}/+; bab:GAL4$^{p4.2}$/+*) was compared to ovariole numbers in GAL4-only or UAS-only parental and sibling controls. We compared samples using a one-way ANOVA followed by Tukey's HSD, and found that in both cases, F1 adult females had significantly more ovarioles compared to parents and siblings ($S6K^{TE}$: $p<0.001$; $S6K^{STDE}$: $p<0.001$). This increase was also reflected in larval TF number ($S6K^{TE}$ $p<0.01$; $S6K^{STDE}$ $p<0.05$) (Fig. 3C).

Constitutively active S6K increases both size and number of TFCs

In *Drosophila* ectodermal tissues (wing, eye) and in mouse ectodermal tissues (adrenal gland) and embryonic fibroblasts, *S6K* activity is linked to control of cell size, but not of cell number (Lawlor et al., 2002; Montagne et al., 1999). However, the function of S6K in mesodermal tissues in *Drosophila* has not yet been investigated. We therefore asked whether the effect on adult ovariole number caused by constitutive *S6K* activity was due to a size change in TFCs. Cell size measurements were taken manually at the larval–pupal transition stage (referred to as "larval–pupal stage" throughout; see Methods) using confocal z-stacks of TFs (Fig. 2A) from four to ten cells per sample (average 7.8; see Materials and methods). F1s (*w; UAS-S6K^{X}/+; babGAL4$^{p4.2}$/+*) and UAS-only controls were compared using a mixed-model nested ANOVA (see Methods). Average TFC size increased in both S6K alleles as compared to controls, although the increase was not statistically significant in the case of the STDE allele ($S6K^{TE}$: $p<0.05$; $S6K^{STDE}$: $p = 0.52$; Fig. 4A, B).

A model that could explain how larger TFCs would result in more terminal filaments, is one where developmental regulation controls total overall TF size. This model predicts that TFs made of larger cells would contain fewer TFCs per TF, in order to maintain constant TF size. To test this model we measured TFC number per TF, and found that it was significantly lower ($p<0.01$) for the $S6K^{TE}$ allele and slightly lower ($p = 0.075$) in the $S6K^{STDE}$ allele (Fig. 4C, D). However, TFC size was not correlated with the number of cells per TF (Fig. S4). This suggests that S6K may have a role in sorting TFCs that is independent of cell size.

The reductions in TFC number per TF were not steep enough to account for the generation of all supernumerary TFs induced by *S6K* constitutive expression. We therefore analyzed the effect of constitutive S6K activity on total TFC number. Surprisingly, expression of both $S6K^{TE}$ and $S6K^{STDE}$ resulted in a significant increase in total TFC number in the experimental cross ($S6K^{TE}$: $p<0.01$; $S6K^{STDE}$: $p<0.05$) (Fig. 4E, F), indicating that constitutively active *S6K* alleles alter cell number in the developing ovary. This contrasts with what has been observed in ectodermal tissues, where *S6K* only affects cell size (Lawlor et al., 2002; Montagne et al., 1999) (Fig. S2). In summary, the increase in ovariole number induced by overexpression of constitutively active *S6K* results from an increase both in TFC size and in cell number. This shows that S6K activity can have cell-type specific effects in *D. melanogaster*.

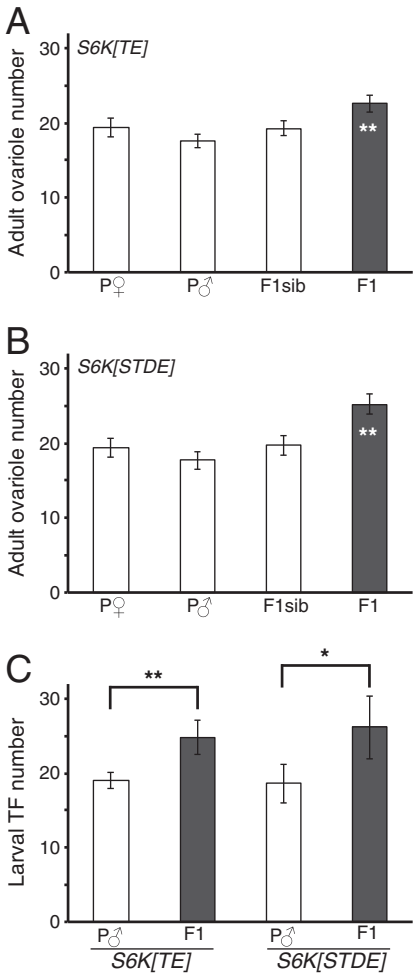

Fig. 3. Expression of constitutively active S6K alleles in TFCs increases ovariole number. (A) Ovariole number in ovaries expressing $S6K^{TE}$ with the *bab:GAL4* driver (Fig. S3) (F1) is significantly higher than that of siblings (F1sib) and both parental strains (P♂, P♀) ($F_{(3,75)}=26.14$, $p<0.0001$), indicated by **. (B) Ovariole number in larval ovaries expressing $S6K^{STDE}$ with the *bab:GAL4* driver (Fig. S3) (F1) is significantly higher ($p<0.01$) than that of siblings (F1sib) and parental strains (P♂, P♀) ($F_{(3,76)}=14.12$, $p<0.0001$), indicated by **. (C) TF number in larval/pupal stage ovaries expressing the different *S6K* alleles (F1) with the *bab:GAL4* driver (Fig. S3) compared with the parental strains (P♂). $n=20$ per genotype for adult ovariole number analysis, and $n=5$ per genotype for larval analysis. In (C) * $p<0.05$, ** $p<0.01$. Error bars indicate 95% confidence interval.

RNAi knockdown of Hippo pathway components in TFCs increases ovariole number

Previous studies had suggested that total ovarian cell number could contribute to ovariole number determination (Hodin and Riddiford, 2000), but did not distinguish between different ovarian cell types. Our cell size manipulation experiments unexpectedly resulted in changes in cell number as well, leading us to suspect that TFC number could be an important parameter in determining TF number. We therefore tested the hypothesis that changes specifically in total TFC number would affect TF number, and hence affect ovariole number. In order to change cell number without changing cell size, we disrupted the activity of the Hippo pathway, a conserved metazoan growth pathway (Dong et al., 2007; Huang et al., 2005; Wu et al., 2003). We used the *bab:GAL4* driver (Fig. S3) and UAS-RNAi strains against two key Hippo pathway kinases, *hippo* (*hpo*) and *warts* (*wts*). RNAi knockdown of these two genes using the same strains from the Transgenic RNAi Project (TRiP) increases proliferation in the gut epithelium (Karpowicz et al., 2010).

RNAi knockdown of *hippo* and *warts* in TFCs (Fig. 2C, D) increased ovariole number of females from the experimental cross (*w*; *UAS:RNAi*/+; *bab:*$GAL4^{p4.2}$/+) compared with GAL4-only and UAS-only parental and sibling controls (Fig. 5A, B). One-way ANOVA revealed a significant difference in ovariole number between these genotypes (*hpo*-RNAi: $p<0.0001$; *wts*-RNAi: $p<0.0001$), and comparisons using Tukey's HSD revealed that in both cases, F1 adult females had significantly more ovarioles compared to parents and siblings ($p<0.05$). This increase was reflected in larval TF number (*hpo*-RNAi: $p=0.028$; *wts*-RNAi: $p=0.037$; Fig. 5C), suggesting that the cellular behaviors underlying the increase in ovariole number take place during larval stages.

Reduced Hippo pathway activity increases total TFC number

To investigate the developmental causes underlying the increase in ovariole number, we then analyzed larval–pupal TFCs with reduced *hpo* and *wts* activity. As expected, TFC cell size was unchanged from controls (*hpo*-RNAi: $p=0.93$; *wts*-RNAi: $p=0.23$; Fig. 6A, B), and we did not observe a difference in TFC number per TF (*hpo*-RNAi: $p=0.58$; *wts*-RNAi: $p=0.72$; Fig. 6C, D). However, there was a significant increase in total TFC number (*hpo*-RNAi: $p<0.01$; *wts*-RNAi: $p=0.028$; Fig. 6E, F). This shows that TF number can be modified by direct changes in total TFC number, without affecting the stacking mechanism that creates TFs. In summary, downregulating the Hippo pathway in TFCs increased total TFC number, thereby increasing the number of TFs created and resulting in higher ovariole number.

Ovariole number differences between D. melanogaster and D. yakuba result from differences in TFC number

Because we found that TFC number was a key regulator of TF number and thus ovariole number in *D. melanogaster*, we hypothesized that evolutionary changes in TFC number could be responsible for ovariole number differences in different *Drosophila* species. To test this hypothesis, we examined TFC number in *D. yakuba* (Fig. 7A, B). This species diverged from the lineage containing *D. melanogaster* 4 to 6 million years ago (Li et al., 1999), and has an average of 14 ovarioles per ovary (Markow and O'Grady, 2007). We first confirmed that this difference in adult ovariole number correlated with a difference in TF number in larval–pupal stage ovaries (Fig. 7C, E, $p<0.05$). Consistent with our hypothesis, this reduced TF number was the result of a smaller total number of TFCs (Fig. 7D, $p<0.01$), which were organized into TFs that contained the same number of TFCs per TF as *D. melanogaster* (Fig. 7E, $p=0.72$). This shows that the developmental basis of evolutionary change in ovariole number between these two species is a change in proliferation of a specific cell population within the ovary, the TFCs.

Adult ovariole number and larval TF number decrease in response to lower rearing temperature or decreased nutrition

Finally, we asked if temperature- and nutrition-dependent phenotypic plasticity in ovariole number could proceed through the same developmental mechanisms as genetic variation. Previous studies reported an effect of temperature on ovariole number in *D. melanogaster*, in both wild and laboratory populations (Chakir et al., 2007; Delpuech et al., 1995; Hodin and Riddiford, 2000; Moreteau et al., 1997). Similarly, nutrient intake can also affect ovariole number in *D. melanogaster*: increasing yeast content in the medium increases ovariole number (Bergland et al., 2008), and relatively reduced nutrient levels results in reduced ovariole number (Hodin and Riddiford, 2000; Robertson, 1957). First, to understand the developmental causes for temperature-induced differences in ovariole number, we analyzed OregonR flies reared at 18 °C and 25 °C on standard fly medium (Fig. S1A). Second, to investigate the developmental basis for nutrition-dependent reduction in ovariole number, we raised OregonR flies at 25 °C on a diet with one quarter the nutrient level of

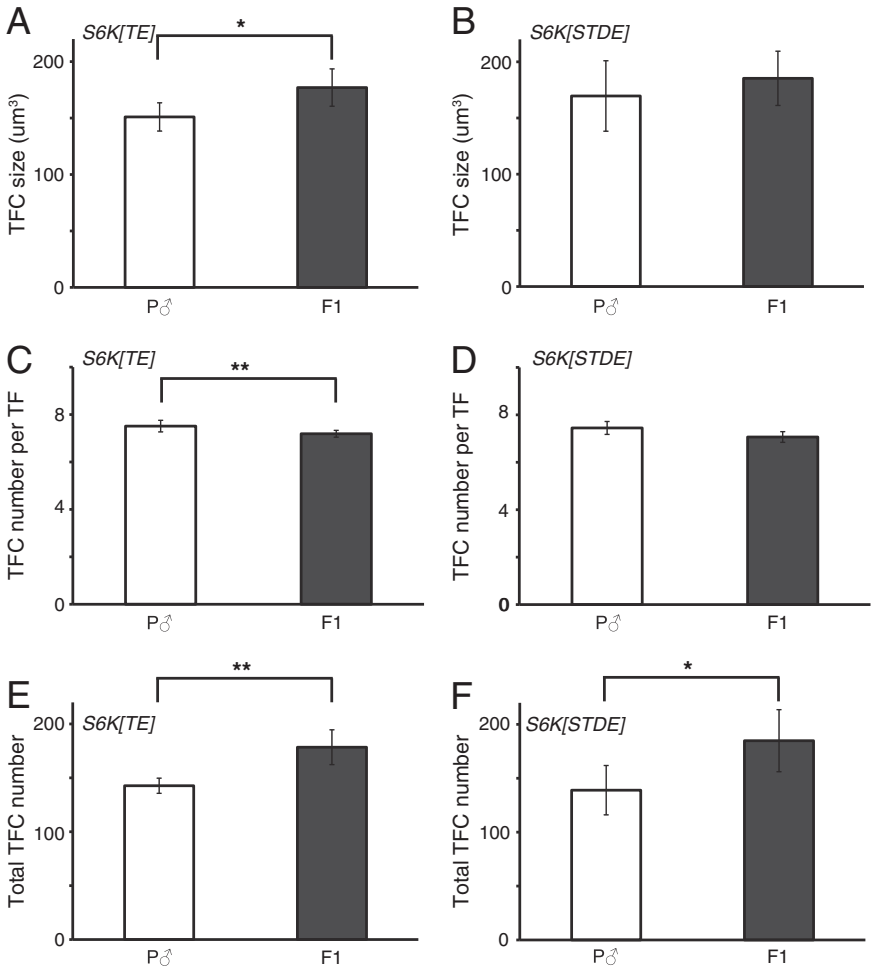

Fig. 4. Expression of constitutively active S6K alleles in TFCs increases TFC cell size and cell number. TFC size in larval–pupal stage ovaries expressing $S6K^{TE}$ (A) and $S6K^{STDE}$ (B) with the *bab:GAL4* driver (Fig. S3) compared with the parental strains (P♂). Total TFC number in larval–pupal stage ovaries expressing $S6K^{TE}$ (C) and $S6K^{STDE}$ (D) compared with the parental strain (P♂). $n=5$ per genotype for analysis. * $p<0.05$, ** $p<0.01$. Error bars indicate 95% confidence interval.

control flies ("quarter food"). In both of these conditions we counted adult ovariole number per ovary and observed, as expected, a significant decrease in ovariole number at 18 °C compared to 25 °C, and on full medium compared to quarter food ($p<0.001$ for both comparisons) (Fig. 8A). Similarly, larval–pupal stage TF number corresponded with adult ovariole number in all conditions (Fig. 8B). The difference was statistically significant in both cases ($p<0.05$ for temperature comparisons and $p<0.01$ for nutrition comparison). This confirms that the decrease in adult ovariole number caused by lower rearing temperature or reducing nutritional intake is a result of reduced larval TF number.

Nutrition affects ovariole number by altering TFC number

We asked whether nutrition, affected ovariole number via the same developmental processes as those altered in our genetic experiments. We found that variation of similar developmental parameters was involved. Flies raised on quarter food had significantly smaller and fewer TFCs than controls (Fig. 8D, E; $p<0001$ in both cases). This is consistent with previous observations that limiting nutrition reduces both cell size and cell number in epithelial tissues (Neel, 1940; Robertson, 1959) (Fig. 8C, S5). However, the number of TFCs per TF was not significantly different between quarter food-raised flies and full food-raised controls (Fig. 8F; $p=0.96$). This indicates that, similar to what we observed when altering cell size with the $S6K^{TE}$ alleles (Fig. 4A, S4A), altering cell size via nutrition does not have a significant impact on TF morphogenesis. The largest contributor to reduced ovariole number in flies raised on quarter-food is therefore the reduction in total TF number (Fig. 8E), which results in fewer TFs being formed.

Rearing temperature does not affect ovariole number by altering TFC number

We then asked whether a second environmental variable, temperature, also changed ovariole number via the same developmental processes as those affected by nutritional deprivation. To do this, we examined TFC size, TFC number per TF, and total TFC number in ovaries of larvae reared at different temperatures. Because temperature correlates negatively with cell size in somatic epithelial tissues (Azevedo et al., 2002) (Fig. 8C), we expected that TFCs would also be enlarged by a colder rearing temperature. Surprisingly however, we found no significant difference in TFC size between the two rearing temperatures ($p=0.58$) (Fig. 8D). As temperature also affects cell cycle and therefore might be expected to change total cell number, we analyzed total TFC number per ovary at 18 °C and 25 °C. In wing cell populations, cell number is not affected by temperature (Azevedo et al., 2002) (Fig. S2). Similarly, no differences were observed in total TFC number between larvae reared at 18 °C and 25 °C (Fig. 8E; $p=0.45$). This demonstrates that, unlike the nutrition-dependent and species-specific differences in ovariole number, the temperature effect on ovariole number is not achieved by changing the number or size of TFCs. Furthermore, the contrast with the temperature effects observed on

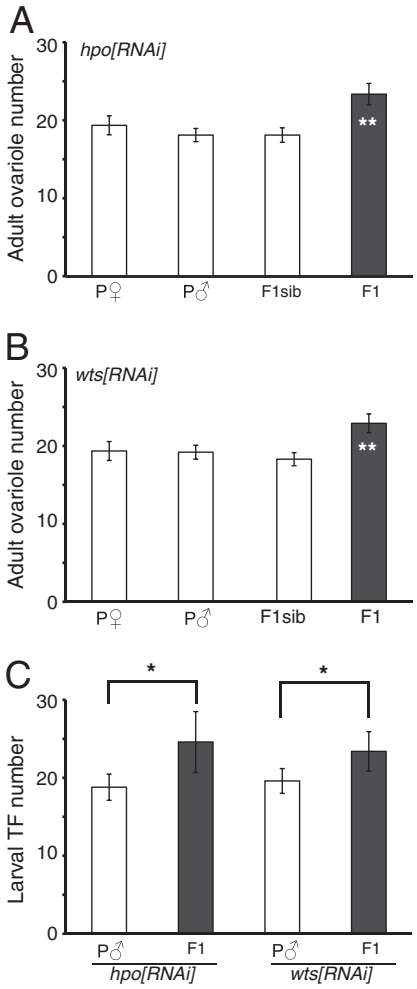

Fig. 5. Decreasing Hippo pathway activity in TFCs increases ovariole number. (A–B) Ovariole number in ovaries expressing *hpo*-RNAi (A) or *wts*-RNAi (B) with the *bab:GAL4* driver (Fig. S3) during development (F1) is significantly higher than F1 siblings carrying only a balancer (F1sib) than both parental strains (P♂, P♀). *hpo*: $F_{(1,80)}=18.16$, $p<0.0001$; *wts*: $F_{(1,80)}=16.29$, $p<0.0001$. (C) TF number in ovaries at larval–pupal stage ovaries expressing the different Hippo pathway RNAi lines (F1) compared with the parental strain (P♂). $n=20$ per genotype for adult ovariole number analysis, and $n=5$ per genotype for larval analysis. * $p<0.05$, ** $p<0.01$. Error bars indicate 95% confidence interval.

wing cell size (Fig. S5) indicates that temperature-induced changes in development can be tissue-specific.

Rearing temperature affects ovariole number by altering TFC number per TF

Even though there was a significant decrease in TF number in larval ovaries reared at 18 °C compared with 25 °C, there was no corresponding significant decrease in total TFC number. This suggested that temperature-induced changes in TF morphogenesis might account for differences in total TF number. Accordingly, we found a significant increase in TFC number per TF in ovaries from larvae reared at 18 °C ($p<0.01$) (Fig. 8F). This suggests that during early ovarian morphogenesis, the size and starting number of TFCs is similar regardless of the temperature. However, as morphogenesis proceeds and TFs form, lower temperatures result in changes to the mechanism that organizes cells, such that a larger number of TFCs are incorporated into each TF. As a result, fewer TFs are formed at lower temperatures.

Discussion

Here we have shown that two distinct developmental mechanisms can alter ovariole number: the establishment of total TFC number (Fig. 9A), and the local cell–cell sorting process during TF formation (Fig. 9B). These two processes appear to be differently employed to alter ovariole number. Specifically, by genetically altering the activity of developmental growth pathways, we observed that change in ovariole number was achieved by changes in total TFC number, rather than by changing TFC number per TF. Similarly, changes in TFC number appeared responsible for ovariole number differences between two *Drosophila* species, and for starvation-induced reduction in ovariole number. In contrast, temperature-induced differences in ovariole number were caused by changes in TFC number per TF, rather than changes in total TFC number or TFC size. We postulate that at least some of the genetic changes underlying species-specific ovariole number may alter total TFC number, while temperature-dependent variation may result from differences in TFC sorting during TF formation.

In this work we have examined specifically the TFC population of the somatic ovary. Previous work has analyzed total ovarian cell number in relation to ovariole number, and did not always find a direct correlation (Hodin and Riddiford, 2000). We therefore suggest that total ovarian cell number is unlikely to be the parameter targeted for evolutionary variation in this trait. Consistent with the hypothesis that total ovarian cell number is not necessarily a useful predictor of ovariole number, *Drosophila mauritiana* has fewer ovarioles than *Drosophila simulans*, but more total ovarian cells than *D. simulans* (Hodin and Riddiford, 2000). Thorax length (a proxy index for body size) can correlate positively with ovariole number, but this correlation is strong only under poor nutritional conditions (Bergland et al., 2008): when grown on food with high yeast concentrations, genetic correlations between thorax length and ovariole number are not significant (Telonis-Scott et al., 2005; Wayne et al., 1997). Our dissection of the response of cellular populations and processes to ovariole number variation suggests that the specific ovarian cell population likely to be the target of evolutionary change is the TFC population. Further studies will be needed to determine if modification of the TFC population is a conserved mechanism of evolutionary change in insect species that differ in ovariole number.

Tissue-specific response to temperature and constitutively active S6K

While investigating the mechanisms underlying ovariole number change, we identified tissue-specific responses to both temperature and overexpression of constitutively active *S6K*. Larval rearing temperature affects overall body size of *D. melanogaster* by causing a change in cell size of the epidermal cells (Azevedo et al., 2002) (Fig. 8C). When the same strain of flies are reared at colder temperatures, the flies are larger, and the cells that compose the epidermal tissues are larger, but there is no difference in cell number. In contrast, we did not observe cell size differences in the ovarian TFC cells, but rather observed a change in the cell–cell sorting behavior during TF formation.

S6K activity in the *Drosophila* wing influences cell size without affecting cell number (Montagne et al., 1999). Our analysis showed that constitutively active *S6K* activity could also increase TFC size, but this increase was only statistically significant with the $S6K^{TE}$ allele. In contrast to the wing, however, constitutive *S6K* activity in TFCs significantly increased cell number. The mammalian *S6K* orthologues S6K1 and S6K2 have been implicated in proliferation in some tissues (reviewed by Fenton and Gout, 2011a), but to our knowledge S6K has not previously been reported to influence cell proliferation in *Drosophila*.

Interestingly, while constitutively active *S6K* significantly increased TFC number, it also decreased TFC number per TF. This was true even for the $S6K^{STDE}$ allele, where cell size was not significantly increased compared to controls. Since a clear correlation between TFC size and number of cells per TF was not observed (Fig. S4), TF size may not contribute significantly to regulating TF number (Fig. 9C). Instead, it is

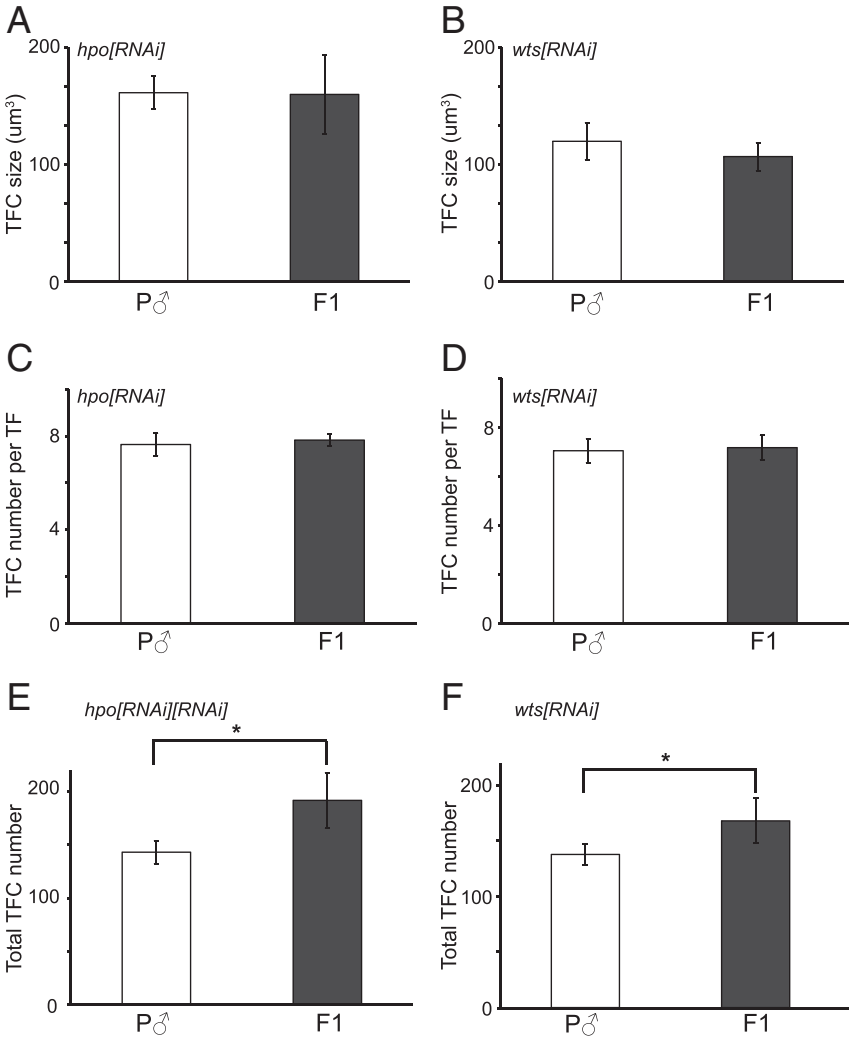

Fig. 6. Decreasing Hippo pathway activity in TFCs increases total TFC number, without affecting cell size or sorting. (A–B) TFC volume of larval–pupal stage ovaries expressing *hpo*-RNAi (A) or *wts*-RNAi (B) with the *bab:GAL4* driver (Fig. S3) compared to parental strain y^1v^1; *UAS-RNAi*. *hpo*: $F_{(1,76)}=0.0066$, $p=0.93$; *wts*: $F_{(1,80)}=1.6$, $p=0.23$. (C–D) TFC number per TF of larval–pupal stage ovaries expressing *hpo*-RNAi (C) or *wts*-RNAi (D) compared to parental strains. *hpo*: $F_{(1,112)}=0.32$, $p=0.58$; *wts*: $F_{(1,102)}=0.13$, $p=0.72$. (E–F) Total TFC number of larval–pupal stage ovaries expressing (E) *hpo*-RNAi and (F) *wts*-RNAi compared to parental strain. $n=5$ per genotype for analysis. * $p<0.05$. Error bars indicate 95% confidence interval.

possible that Insulin or TOR signaling, which both act via S6K (reviewed by Fenton and Gout, 2011a), may also be involved in the process of cell–cell sorting of TFCs.

The developmental mechanisms influencing evolutionary change in ovariole number

The ovaries of all insects are composed of ovarioles, and ovariole number changes frequently in insect evolution (Büning, 1994). One of the best-studied examples of ovariole number change is in honeybees, where females develop into queens with hundreds of ovarioles, or workers with only five to ten ovarioles, depending on larval nutrition (Haydak, 1970). The developmental process that ultimately results in ovariole number difference between queens and workers is increased apoptosis in worker ovaries during late larval instars, which actively reduces ovarian structures, and higher ovarian cell proliferation in queens (Reginato and Cruz-Landim, 2001; Reginato and Cruz-Landim, 2003; Reginato and da Cruz-Landim, 2002). However, it is unclear which specific cell population is the dominant contributor to either apoptosis or proliferation in shaping honeybee ovariole number (Capella and

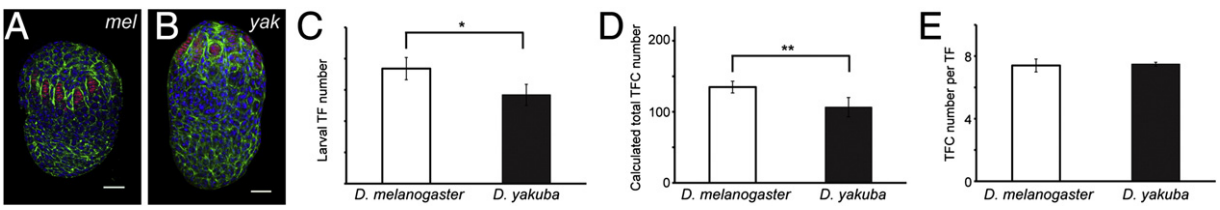

Fig. 7. TF number, TFC number and TF morphogenesis in *D. yakuba*. Larval–pupal stage ovaries in *D. melanogaster* (A) and *D. yakuba* (B). (C) TF number at larval–pupal stage of *D. yakuba* and *D. melanogaster* . (D) TFC number at larval–pupal stage in *D. yakuba* and *D. melanogaster*. (E) TFC number per TF at larval–pupal stage in *D. yakuba* and *D. melanogaster* ($F_{(1,148)}=0.1323$, $p=0.72$). Animals were reared at 25 °C for all experiments. In (A, B) anterior is up and scale bar = 20 μm. * $p<0.05$, ** $p<0.01$. Error bars indicate 95% confidence interval. Apparent morphological differences between (A) and (B) are an artifact of flattened preparation in (B).

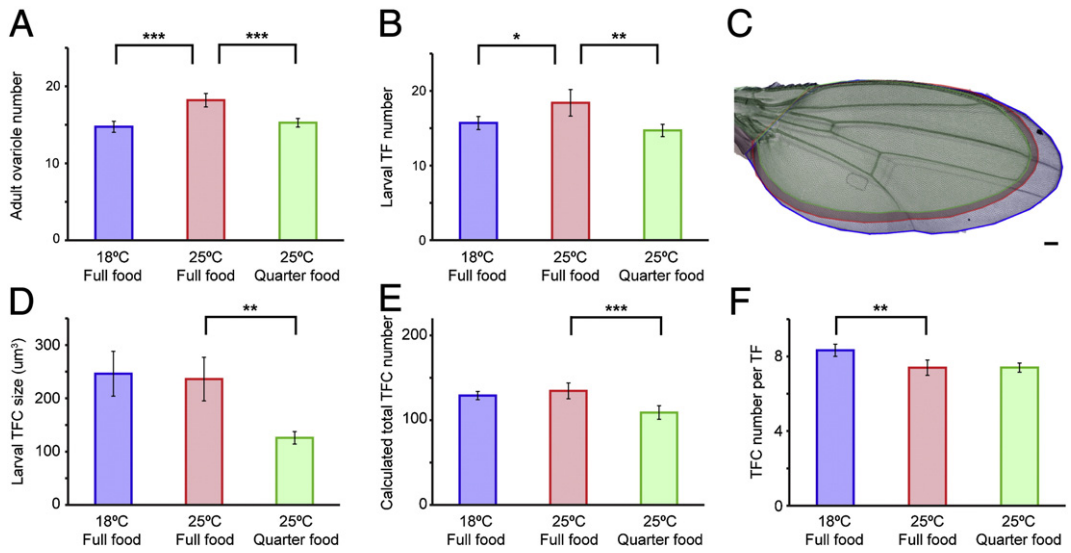

Fig. 8. The effects of temperature and nutrition on ovariole number, TF number and TFC morphogenesis. (A) Ovariole number decreases at colder temperatures and in flies raised on quarter food. Mean ovariole number of OregonR flies reared at 18 °C (blue), 25 °C (red) and on quarter food (green). (B) TF number at larval–pupal stage of animals reared at 18 °C, 25 °C and on quarter food. (C) Wing size increases at colder temperatures and decreases with reduced nutrition. Outlines of total adult wing surface area of flies reared at 18 °C, 25 °C, and on quarter food; n = 5 for all conditions. Scale bar = 100 μm. Anterior is to the left. (D) TFC size in larval–pupal stage OregonR ovaries reared at 18 °C, 25 °C, and on quarter food (between temperatures $F_{(1,57)} = 0.3288$, $p = 0.58$; between nutritional regimes $F_{(1,177)} = 25.69$, $p < 0.001$). (E) Total TFC number of larval–pupal stage OregonR ovaries reared at 18 °C, 25 °C and on quarter food (between temperatures $p = 0.45$; between nutritional regimes $p < 0.001$). (F) TFC number per TF of larval–pupal stage OregonR ovaries reared at 18 °C, 25 °C and on quarter food between temperatures $F_{(1,182)} = 12.22$, $p < 0.01$; between nutritional regimes $F_{(1,67)} = 0.0019$, $p = 0.96$). $n = 40$ adults per temperature for adult ovariole counts, and $n = 10$ larvae per temperature for TF number counts. Error bars indicate 95% confidence interval. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Hartfelder, 1998; Hartfelder and Steinbruck, 1997; Reginato and da Cruz-Landim, 2002). In contrast to honeybees, apoptosis is not a regulator of ovariole number in *Drosophila* species (Hodin and Riddiford, 2000), but our now data demonstrate that higher TFC proliferation can also increase ovariole number in *D. melanogaster*. This suggests that proliferation control of the TFC population may be a developmental process that is a target of evolutionary change in ovariole number in *Drosophila*, and perhaps in other insects.

Ovariole number in Drosophilid flies can change relatively rapidly within a clade. For example, the *melanogaster* subgroup contains *D. simulans*, *D. mauritiana*, and *Drosophila sechellia*, three species that diverged from a common ancestor less than one million years ago., Their species-specific ovariole numbers are approximately 35, 28, and 17 respectively, and are proportional to fecundity: *D. simulans* is the most fecund of these three species, and *D. sechellia* the least, under standard laboratory rearing conditions (R' kha et al., 1997). These Drosophilids do not display a difference in apoptosis in the developing ovary, but rather have different numbers of total ovarian cells in late larval stages (Hodin and Riddiford, 2000), consistent with the differences in total TFC number that we observed here for *D. melanogaster* and *D. yakuba*. This further supports our hypothesis that TFC proliferation, rather than differential apoptosis, is a

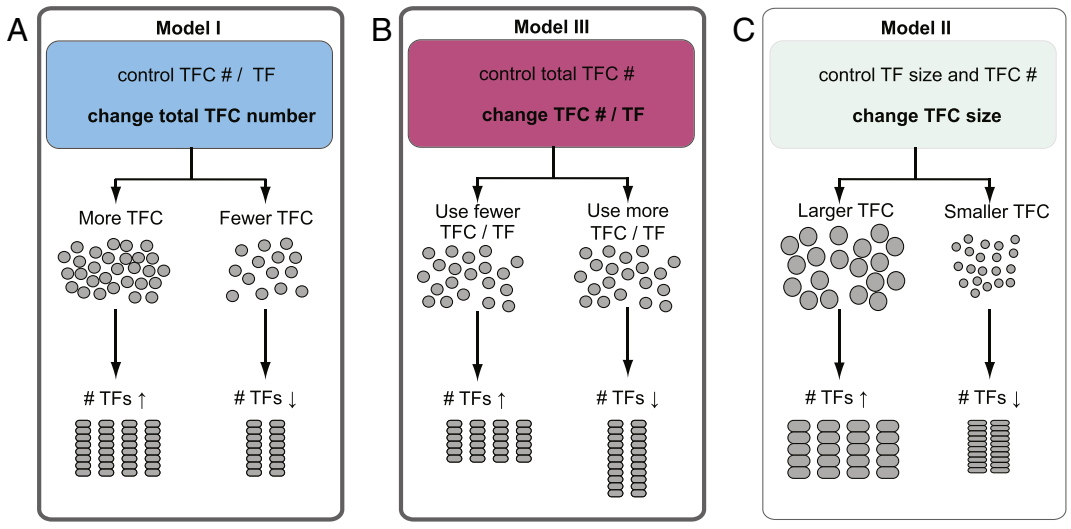

Fig. 9. Models for developmental parameters that determine ovariole number. (A) Model I: TFC number determines TF number. This model predicts that ovariole number variation is achieved through changes in total TF cell number, but not by altering TF cell number per TF. (B) Model II: TFC number per TF determines TF number. Under this model, variation in ovariole number occurs by changes in TF Cell number per TF, regardless of total TF cell number. (C) Model III: TF morphogenesis controls for TF size. This model predicts that the TF morphogenesis program detects and controls overall TF size; changes in ovariole number would therefore come about through differences in TFC size. This would be predicted to have a secondary effect on TF cell number per TF, such that TFs with larger cells would have fewer cells per TF; however, our data do not support this corollary of Model III (Figure S4). Our data suggest that growth pathway activity can affect ovariole number primarily via Model I and to a lesser extent via III, and that both nutritional intake and genetic variation between species may change ovariole number via these developmental mechanisms. In contrast, temperature effects on ovariole number in *D. melanogaster* proceed via Model II.

developmental process subject to evolutionary change in Drosophilid ovariole number. Because developmental studies on this group have thus far been limited, future work could take advantage of this clade as an opportunity to study the developmental basis for ovariole number variation across shorter evolutionary time scales.

Cell types and evolutionary change

Evolutionary change in *Drosophila* wing size occurs through changes in both cell number and cell size, where selective pressures are proposed to act on the size of the entire wing, rather on specific mechanisms of cell proliferation or growth (Zwaan et al., 2000). Dipteran wing development comprises a continuous, interlocked set of processes, in which proliferation, growth and patterning of all wing disk cells show a high degree of coupling throughout development (Baena-Lopez and Garcia-Bellido, 2006; Garcia-Bellido and Garcia-Bellido, 1998; Rafel and Milan, 2008; Resino and Garcia-Bellido, 2004). By contrast, in ovariole development discrete steps of proliferation, patterning, movement and sorting by one of many distinct ovarian cell types are required to produce TFs. Each step of TF formation is relatively autonomous with respect to the behaviors of other ovarian cell types during morphogenesis, and to global body-wide processes of growth and patterning (Green & Extavour, unpublished observations; Boyle and DiNardo, 1995; Gilboa and Lehmann, 2006; Kerkis, 1931; King, 1970; Li et al., 2003; Riechmann et al., 1998). TFC behavior may thus be able to change in response to a particular evolutionary pressure, without large effects on the other aspects of ovarian or general somatic development. In this context, *Drosophila* ovaries provide an interesting model for addressing the role of different cell types in organ size evolution.

In summary, we have taken a developmental approach to a long-standing question regarding the evolution of a quantitative fitness trait, and shed new light on the specific cell population likely to be the target of evolutionary change in ovariole number. We hypothesize that the most promising candidate pathways for future investigation of species-specific genetic changes affecting ovariole number are pathways that control growth and cell proliferation in TFCs. These may include cell cycle genes, long-range signaling molecules, and organ-level proliferation and growth control pathways. Consistent with this hypothesis, several such genes, including the insulin receptor, are contained in the *Drosophila* QTL that have been identified as linked to inter- and intraspecies variation in this trait (Orgogozo et al., 2006; Wayne and McIntyre, 2002; Wayne et al., 2001), and insulin pathway genes are present in some honeybee QTL linked to ovariole number differences (Hunt et al., 2007). Intriguingly, differential activity of the insulin pathway can alter ovariole number in both *D. melanogaster* (Green & Extavour, unpublished observations; Richard et al., 2005; Tu and Tatar, 2003) and in honeybees (Mutti et al., 2011; Patel et al., 2007; Wolschin et al., 2011). Our work provides novel developmental and cell biological tools to test the hypotheses that these and other genes have been the direct targets of evolutionary change leading to ovariole number variation.

Supplementary materials related to this article can be found online at doi:10.1016/j.ydbio.2011.12.017.

Author contributions

CE conceived the idea for the research; AAB (initial S6K analyses), AA (*hpo:RNAi* analyses of TF number and TFC/TF number), DAG (analysis of the *bab:GAL4* expression pattern), AD (*D. yakuba* analysis) and DS (all other experiments) performed experiments and collected data; CE, AAB and DS analyzed data; CE and DS wrote the paper with input from AA and DAG; CE obtained funding for the research.

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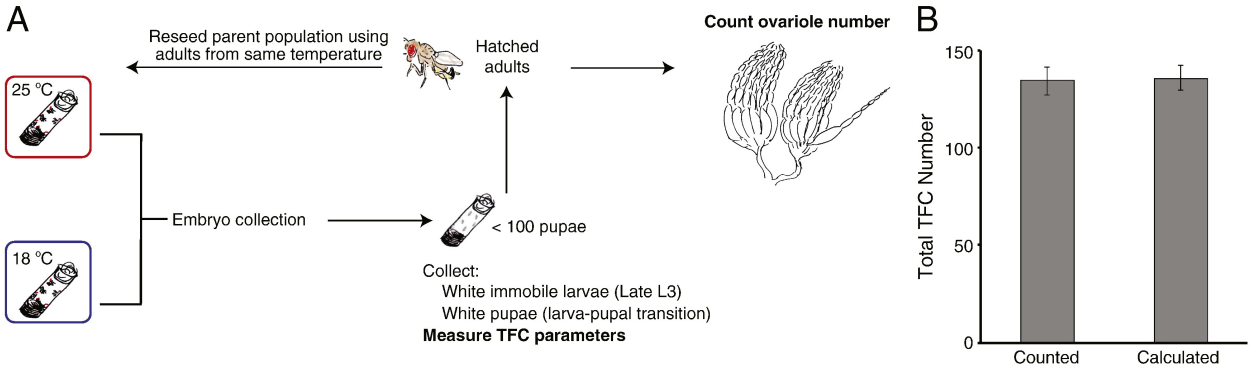
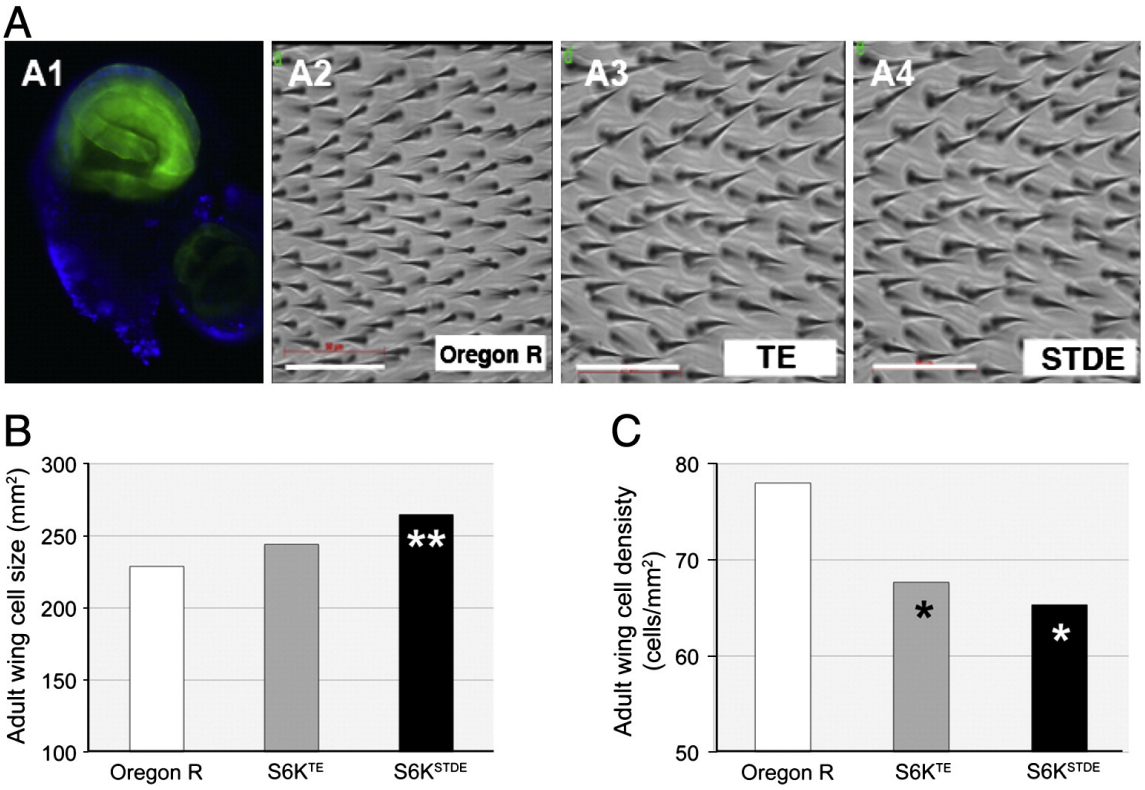
Supporting Materials for

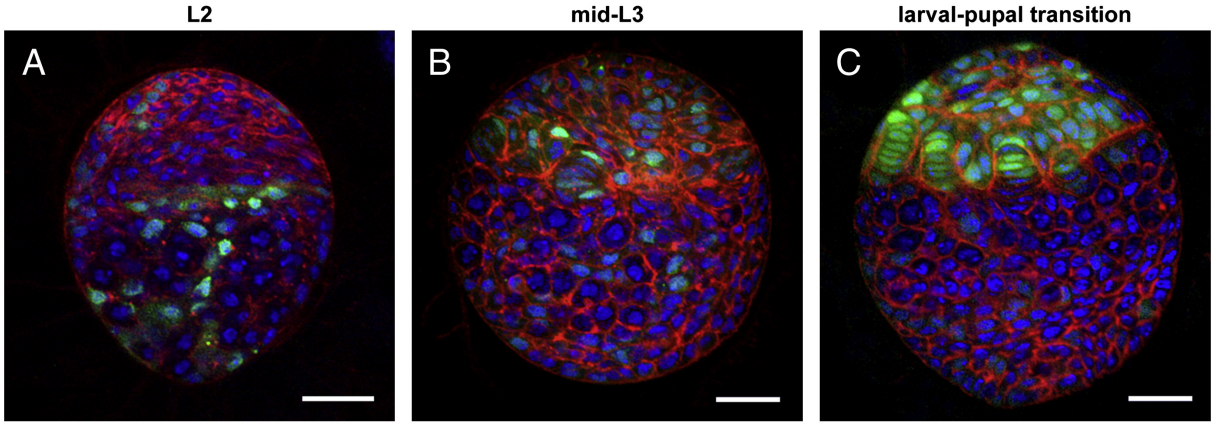
The roles of cell size and cell number in determining ovariole number in *Drosophila*.
Sarikaya, D.P., Belay, A.A., Ahuja, A., Green, D.A., Dorta, A. and **Extavour, C.G.**,
Developmental Biology 363(1): 279-289 (2012)



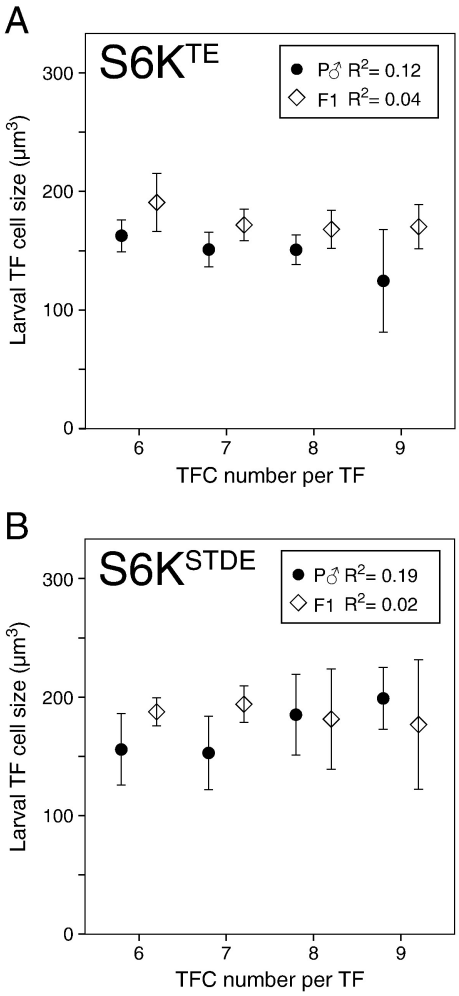
Supplementary Fig. 1. Experimental design. (A) For temperature controlled experiments, flies were reared at 25 °C or 18 °C at 60% humidity on standard fly media for at least two generations before experiments were conducted. Adults were permitted to lay eggs in vials for two to six hours and then removed from the vial to prevent overcrowding of larvae (Fig. S1A). Only tubes containing fewer than 100 pupae were used for analysis of larval–pupal ovaries, and for counts of ovariole number in adults. Adults hatched from these tubes were used to create new parent cultures at the same temperature. (B) Comparison of the mean of calculated and manual counts of total TFC number per ovary. To test whether calculated TFC numbers would accurately represent the total TFC number, calculated TFC numbers were obtained by randomly choosing five ovaries where all TFC per TF were counted, and multiplying the average TFC number per TF (obtained from the randomized five data points) by the total number of TFs in the ovary. This was compared to manual counts of total TFC numbers of the same ovaries. Differences between counted and calculated total cell numbers did not exceed ± 4 and were not significantly different ($p = 0.79$). Error bars indicate 95% confidence interval.



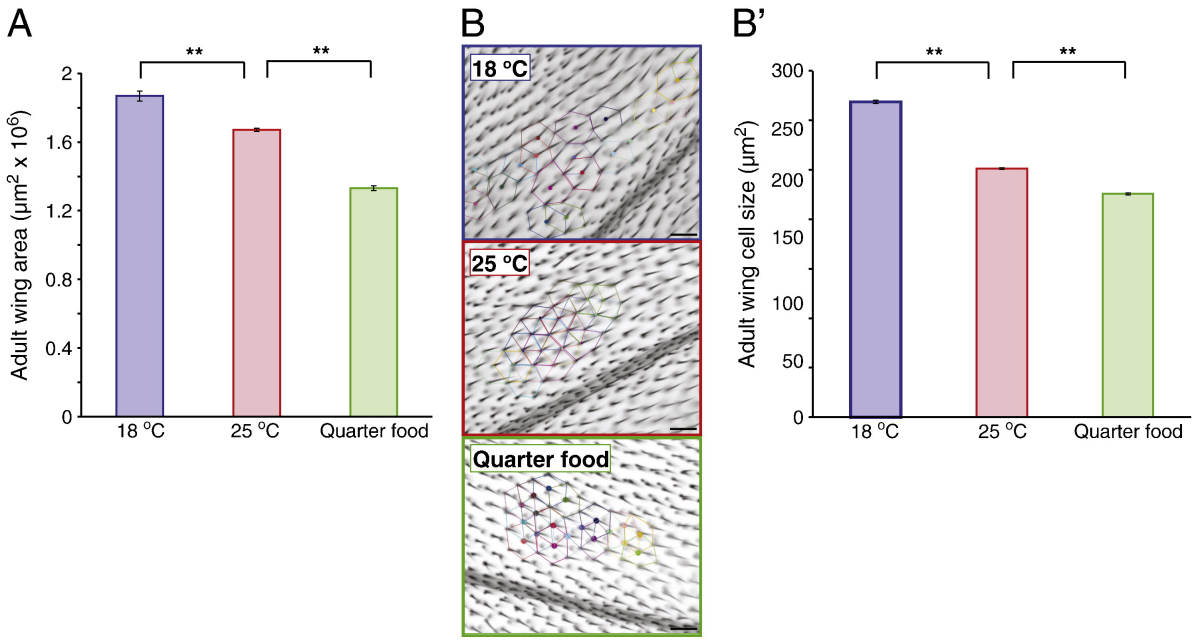
Supplementary Fig. 2. Constitutively active *S6K* alleles increase wing cell size but do not affect cell number. (A) A *nub:GAL4*driver was used to express constitutively active alleles of *S6K* in the wing pouch. Anterior is up. (A1) The expression domain of the *nub:GAL4* driver revealed by UAS:GFP. Driver expression in the wing imaginal disk is confined to the wing pouch, which gives rise to the wing proper (but not the notum). Green: GFP; blue: Hoechst. (A2) Trichomes in the wing of a wild type adult female, compared with those in the wings of adult females whose wings express *S6KTE* (A3) or *S6KSTDE* (A4). A2–A4 are at the same magnification; scale bar = 50 μm. (B) Adult wing cell size is larger than controls in wings expressing *S6KTE*, and significantly larger ($p < 0.01$) in wings expressing *S6KSTDE*. (C) Consistent with increased cell size, adult wing cell density is significantly smaller than controls in wings expressing either *S6KTE* or *S6KSTDE* ($p < 0.05$). Total wing cell number is not affected by these mutations (Montagne et al., 1999 and data not shown).



Supplementary Fig. 3. *bab:GAL4* is expressed in TFCs throughout larval development. Expression of GFP driven by *bab:GAL4*during larval ovarian development (A) Second larval instar (L2). (B) Mid-third larval instar (L3). (C) Larval–pupal stage. Green: GFP; red: phalloidin; blue: Hoechst. Anterior is up. Scale bar = 20 mm.



Supplementary Fig. 4. Number of cells per TF is not correlated with TFC size. Size of TFCs at late L3 larval stages as a function of TFC number per TF, in ovaries overexpressing *S6KTE* (A) or *S6KSTDE* (B) compared to parental strains (P♂).Error bars indicate 95% confidence interval.



Supplementary Fig. 5. Effects of temperature and nutrition on wing cell size, wing size, and wing cell number. (A) Measurements of wing surface area of flies reared at 18 °C (blue), 25 °C (red) and on quarter food (green). Measurements were made of the same wings shown in outline in Fig. 8C. Wings of flies reared at 18 °C are significantly larger than wings of flies reared at 25 °C, and wings of flies reared on quarter food are significantly smaller ($p < 0.001$ for both comparisons). (B) Wing cell size (surface area) was measured by halving the area of the region delimited (colored lines) by the six neighbors of a given microchaete (wing hair), since every wing cell secretes a single microchaete. Scale bar = 20 μm. (B′) Measurements of wing cell size of flies reared at 18 °C and at 25 °C. Wing cells of flies reared at 18 °C are significantly larger than those of flies reared at 25 °C, and wing cells of flies reared on quarter food are significantly smaller ($p < 0.001$ for both comparisons). Error bars indicate 95% confidence interval.