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Evolution and Developmental Control Mechanisms

Convergent evolution of a reproductive trait through distinct developmental mechanisms in *Drosophila*

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ABSTRACT

Convergent morphologies often arise due to similar selective pressures in independent lineages. It is poorly understood whether the same or different developmental genetic mechanisms underlie such convergence. Here we show that independent evolution of a reproductive trait, ovariole number, has resulted from changes in distinct developmental mechanisms, each of which may have a different underlying genetic basis in *Drosophila*. Ovariole number in *Drosophila* is species-specific, highly variable, and largely under genetic control. Convergent changes in *Drosophila* ovariole number have evolved independently within and between species. We previously showed that the number of a specific ovarian cell type, terminal filament (TF) cells, determines ovariole number. Here we examine TF cell development in different *Drosophila* lineages that independently evolved a significantly lower ovariole number than the *D. melanogaster Oregon R* strain. We show that in these *Drosophila* lineages, reduction in ovariole number occurs primarily through variations in one of two different developmental mechanisms: (1) reduced number of somatic gonad precursors (SGP cells) specified during embryogenesis; or (2) alterations of somatic gonad cell morphogenesis and differentiation in larval life. Mutations in the *D. melanogaster* Insulin Receptor (*InR*) alter SGP cell number but not ovarian morphogenesis, while targeted loss of function of *bric-à-brac 2* (*bab2*) affects morphogenesis without changing SGP cell number. Thus, evolution can produce similar ovariole numbers through distinct developmental mechanisms, likely controlled by different genetic mechanisms.

Introduction

Convergent morphologies can evolve independently in different lineages, often as a result of similar selective pressures or functional requirements. An outstanding question in evolutionary and developmental biology is whether similar traits evolve convergently through changes in the same or different developmental and genetic processes. Changes in different processes imply that natural selection can act on multiple developmental processes to achieve the same outcomes, whereas changes in the same processes may suggest that natural selection is constrained to act on one developmental event (Losos, 2011; Sanger et al., 2012). In recent years, several examples of convergent evolution at the molecular, cellular and morphological levels have been examined (Aminetzach et al., 2009; Moczek et al., 2006; Protas et al., 2006; Sucena et al., 2003; Tanaka et al., 2009; Wittkopp et al., 2003). In some of these cases, similar morphologies have evolved independently via changes in the same genes or genetic pathways (Chan et al., 2010; Protas et al., 2006; Prud'homme et al., 2006; Sucena et al., 2003; Zhang et al., 2012). However, in other cases convergent evolution of similar traits arises through different developmental or genetic mechanisms (Moczek et al., 2006; Shapiro et al., 2009; Steiner et al., 2009; Tanaka et al., 2009; Wittkopp et al., 2003; Zwaan et al., 2000).

In many of the cases where the genetic basis is well understood, the convergent trait hinges on the terminal differentiation of a single cell type, such as adult cuticle pigmentation (Gompel and Carroll, 2003; Prud'homme et al., 2006; Steiner et al., 2009; Wittkopp et al., 2003) or larval hairs (Sucena et al., 2003) in *Drosophila*. However, there are few well-studied examples in which the convergent trait involves a multicellular structure composed of many distinct cell types (Moczek et al., 2006; Tanaka et al., 2009; Zwaan et al., 2000). Moreover, while many external anatomical traits have been studied in this context, the evolution of internal reproductive morphologies that directly affect fecundity are less well understood. As a step towards elucidating the genetic mechanisms underlying the evolution of reproductive morphologies, here we examine changes in development that lead to major differences in ovariole number, an aspect of ovarian morphology that directly affects egg production and reproductive capacity in *Drosophila*.

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All insect ovaries are composed of ovarioles, which are egg-producing substructures of the ovary. Ovariole number is positively correlated with egg production and fecundity (David, 1970), suggesting that this trait can have a significant impact on fitness and is likely to be under selective pressure. Ovariole number also varies across latitudinal (Boulétreau-Merle et al., 1992; David and Bocquet, 1975; Delpuech et al., 1995; Gibert et al., 2004; Paaby et al., 2010; Schmidt et al., 2005) and altitudinal (Collinge et al., 2006; Wayne et al., 2005) clines, further suggesting that this trait may be locally adaptive. Ovariole number variation across insects is dramatic, ranging from fewer than five per ovary in some flies to hundreds per ovary in crickets and grasshoppers (Büning, 1994).

Ovariole number has been the subject of extensive ecological and quantitative genetic studies for decades (reviewed in Hodin, 2009). Albeit not to single-gene resolution, these investigations have shown that ovariole number is a polygenic trait (Coyne et al., 1991; Thomas-Orillard, 1976), and inter- and intraspecific ovariole number variation is linked to changes at numerous loci (Bergland et al., 2008; Orgogozo et al., 2006; Telonis-Scott et al., 2005; Wayne et al., 2001; Wayne and McIntyre, 2002). Determining promising candidate genes from these QTL studies is difficult, because ovarian morphogenesis is relatively poorly understood, and only a small number of genes have been shown to play a specific role in ovariole formation (Gancz et al., 2011; Godt and Laski, 1995; Hodin and Riddiford, 1998). Thus candidate genes within these loci have not yet been functionally investigated for causal links to ovariole number. We hypothesized that a better understanding of the cellular and developmental mechanisms governing ovariole formation would help to identify candidate genes that may underlie ovariole number evolution.

Ovariole morphogenesis begins with the formation of stacks of somatic cells, called terminal filaments (TFs), in the anterior of the larval ovary (Godt and Laski, 1995) (Fig. 2A). Each TF is the starting point for the development of one ovariole, such that ovariole number is directly determined by TF number (Hodin and Riddiford, 2000). We previously showed that a major determinant of TF number is the total number of TF precursor cells present in the larval ovary before TF formation begins, and that TF cell number varies between *Drosophila* species with different ovariole numbers (Sarikaya et al., 2012). Here we explore even earlier developmental processes to understand why *Drosophila* lineages have different TF cell numbers, and whether TF cell number variation can explain differences in ovariole number in a broader range of Drosophilids.

In this study we analyze and compare the process of ovarian morphogenesis in *Drosophila* lineages that independently evolved a significantly lower average ovariole number than the North American *D. melanogaster Oregon R* (*OR*) strain: the *D. melanogaster* "India" strain (*Ind*) and the single-niche specialist *D. sechellia* "Robertson" strain (*Ds*) (Table 1, Fig. 1) (Markow and O'Grady, 2007). One hypothesis for the adaptive significance of lower ovariole number may be its positive correlation with larger egg size that often accompanies ecological specialization in *Drosophila* and other flies (Kambysellis et al., 1995; Markow et al., 2009; Rkha et al., 1997), and could potentially lead to higher hatching rates or larval fitness (Azevedo et al., 1997). We show that similar TF cell numbers and therefore similar ovariole numbers are achieved in these lineages by changes in very different developmental processes. Establishing a smaller pool of somatic gonad cells during embryogenesis in *Ds*, or changing morphogenesis of specific ovarian cell types during larval development in *Ind*, both result in lower ovariole numbers than in *OR*. By analyzing the development of different ovarian cell types in these lineages, we demonstrate that within the same organ, evolutionary changes occur independently in different cell types. We use functional analysis in *D. melanogaster* to show that different genetic pathways influence these distinct developmental mechanisms. Our results show that major changes in reproductive capacity can evolve via distinct developmental mechanisms among closely related lineages.

Table 1
Ovariole number of wild type *Drosophila* strains used in this study.

Species	Strain	Average ovariole number per ovary	Standard deviation	n[a]
D. melanogaster[b]	*Oregon R* (*OR*)	18.2	2.8	40
	w^{1118}	18.6	2.1	22
	Nevada-04	18.4	2.2	22
	RAL-301	22.1	2.3	22
	Catalina Island, CA	22.6	2.3	22
	Santa Fe, NM	22.5	2.2	22
	India (*Ind*)	12.2	1.4	22
	France	16.3	1.8	20
***D. sechellia* (*Ds*)**	14021-0248.25[c]	7.6	1.0	54
D. yakuba[d]	1402–0261.01	14.2	1.9	5

[a] *n* indicates total number of individual adult ovaries in which ovariole numbers were counted.
[b] All *D. melanogaster* strains derive from flies originally collected in North America except for the *India* and *France* strains.
[c] Strain numbers indicate stock numbers from the *Drosophila* Species Stock Center.
[d] Numbers shown for *D. yakuba* indicate terminal filament number, which approximately equals adult ovariole number as shown previously (Sarikaya et al., 2012; Hodin and Riddiford, 2000) and in this study. These numbers also correspond with previously reported numbers for average ovariole number in this species (Markow et al., 2009).

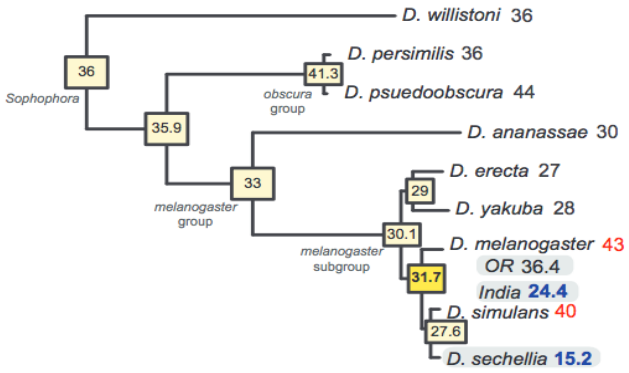

Fig. 1. Reduced ovariole number has evolved independently in *Ds* and *Ind*. Ancestral state reconstruction of ovariole number across members of the genus *Drosophila*. Maximum likelihood values are indicated in boxes at nodes. Lineages analyzed in this study are highlighted in gray. The node representing the last common ancestor of *OR*, *Ind*, and *Ds* is indicated in boldface. Significant increases or decreases in average ovariole number relative to ancestral values are indicated in red and blue, respectively. For 95% confidence intervals see Table S1.

Materials and methods

Drosophila strains and mutant stocks

D. melanogaster OregonR-C (Bloomington *Drosophila* Stock Center (BDSC) ♯5), *D. yakuba* (*Drosophila* Species Stock Center (DSSC) ♯1402–0261.01) and *D. sechellia* Robertson strain (DSSC ♯14021-0248.25) were obtained from the Hartl lab (Harvard

University). North American *D. melanogaster* strains (isofemale lines derived from females collected in respective locations) obtained from the DePace lab (Harvard Medical School) were Nevada-04 (*NV*), Raleigh-301 (*NC*), Catalina Island (CA), and Sante Fe (*NM*). The *D. melanogaster India* (DSSC ♯14021-0231.06) and *France* strains were a gift of the Ludwig lab (University of Chicago). Other BDSC stocks used were the hypomorphic *InR* alleles InR^{E19} (♯9646) and InR^{GC25} (♯9554) and *w*; *P{w[+mW.hs]=GawB}bab1*$^{Pgal4-2}$*/TM6B Tb*^{1} (referred to as *bab-GAL4* in the text; ♯6803) (Cabrera et al., 2002).The *bab2* RNAi line *w*; *bab2-RNAi* (Transformant ID ♯49042) was obtained from the Vienna *Drosophila* RNAi Center.

Ancestral state reconstruction

Maximum likelihood estimates of ancestral character state and associated 95% confidence intervals at each internal node were derived using the Analysis of Phylogenetics and Evolution (APE) package in R (Paradis et al., 2004). The phylogenetic tree and branch lengths, derived from synonymous substitution rates in 12 Drosophilids, are from Heger and Ponting (2007). The ovariole numbers used in these analyses are from Markow et al. (2009).

Culture conditions and larval staging

Drosophila stocks were maintained at 25 °C at 60% humidity under optimal nutrition and without crowding as previously described (Sarikaya et al., 2012). For larval staging analyses, eggs were collected overnight on medium (supplemented with a 1 cm^2 piece of filter paper soaked in N-caprylic acid (Sigma) for *D. sechellia*) in 6 cm-dish collection chambers. 18–22 h after collection start, dishes were cleared of adult flies and hatched larvae. Newly hatched larvae were collected 2 h after clearing and transferred to fresh vials containing standard medium (<100 larvae per vial), establishing L0 ($\pm$ 1 h) larvae. At each time point, body size was used to guide selection of appropriately developed larvae. Larval–pupal transition (LP) stage larvae were identified as previously described (Ashburner et al., 2005).

Adult analysis: ovariole number and body size

Adult ovariole number was determined as previously described (Sarikaya et al., 2012). Gonads from individuals of a particular genotype or stage were pooled and remained so through mounting. Although not eliminated, the possibility of counting ovaries from the same individual was reduced by mounting samples from significantly more individuals than ovaries analyzed. ANOVA analysis confirmed that variance was not significantly artificially decreased by within-individual correlation (not shown). Adult tibia length was used as a proxy for adult body size (Macdonald and Goldstein, 1999). Images were taken using a Zeiss AxioImager Z1 and a Zeiss MRm AxioCam driven by AxioVision v4.6. Measurements were performed as previously described using Image J (v.1.45) software.

Larval analysis: TFC number per TF, TFC number, and TFC size

These parameters were determined as previously described (Sarikaya et al., 2012) with the modification that optical confocal sections were captured at 0.9–1.2 $\times$ zoom in 0.5-2.5 µm thick sections spanning the entire ovary, and analyzed using Image J (v.1.45) software. When reporting LP stage somatic cell proportioning, 'TF cells' measurements include a small proportion of cells that will adopt cap cell fate. As reported by Godt and Laski (1995), cap cell number per niche/TF averages 2.5. We observed similar values in lineages reported here (*average*=2.85 (standard deviation (sd)=0.75), 2.71 (*sd*=0.86), and 2.82 (*sd*=0.79) for *OR*, *Ds*, and *Ind*, respectively), with no statistically significant differences among lineages ($p=0.561$, *OR* vs. *Ds*; 0.877, *OR* vs *Ind*; 0.648, *Ds* vs *Ind*). Cap cells were identified as cells with lower nuclear Engrailed (compared to strong expression in TF cells), rounded (versus flattened) nuclei, and directly adjacent to Vasa-expressing germ cells. Statistical comparisons between sample means were made using a two-tailed Student's *t*-test, and comparisons of mean cell proportions were made using Pearson's chi-squared test.

Larval analysis: total cell number

Total cell number was counted using a methodology similar to that used for TFC number counts. At the LP stage, "anterior somatic cells" are somatic cells located anterior to the germ cells, and include TFCs and apical cells, which were distinguished by the presence (TFC) or absence (apical) of Engrailed expression. In a few cases, cells adjacent to germ cells were also counted as anterior (apical) somatic cells if their nuclei were elongated along the A–P axis, as these cells are apical cells that are migrating posteriorly to delineate individual ovarioles. All other somatic cells were called "posterior somatic cells," the majority of which result from swarm cell migration, which is nearly complete by the LP stage. Swarm cells prior to late-third instar stages were identified by morphology and location within the ovary relative to other cell types. Germ cells were identified by Vasa expression.

Larval analysis: ovary volume

Ovary volume was approximated by measuring the volume of all ovarian nuclei using Volocity (v.4, Perkin Elmer) to define "objects" as those points exceeding 7.5% intensity level (empirically determined to be the optimal intensity value) in the Hoechst channel. For this analysis, images of ovaries were obtained using similar, but not identical, confocal settings. However, the use of *percent* intensity, versus *absolute* intensity, to identify objects controls for acquisition differences. Objects smaller than 10 μm^3 were discarded. The largest object identified was recorded as the ovary volume approximation. The volumes of additional objects were added to the largest volume if the object was $>1\%$ the volume of the largest object.

Immunohistochemistry

Immunostaining was carried out as previously described (Sarikaya et al., 2012). The following primary antibodies were used: mouse 4D9 anti-Engrailed (1:40, Developmental Studies Hybridoma Bank), guinea pig anti-Traffic jam (1:30,000, gift of D. Godt, University of Toronto), rabbit anti-Vasa (1:500, gift of P. Lasko, McGill University). Secondary reagents used were Hoechst 33342 (Sigma, 1:500 of 10 mg/ml stock solution), goat anti-mouse Alexa 568, goat anti-guinea pig Alexa 488, and donkey anti-rabbit Alexa 647 (1:500, Invitrogen). Samples were mounted in Vectashield (Vector labs) and imaged using a Zeiss LSM 710 confocal microscope.

Results

Reduced ovariole number convergently evolved in Ds and Ind

Ovariole number is highly variable among the Drosophilids (reviewed by Hodin, 2009). Although ovariole number is phenotypically plastic and can vary due to different environmental or nutritional conditions (Capy et al., 1993; Kambysellis and Heed,

1971; Sarikaya et al., 2012), under constant environmental conditions it falls within a heritable, species-specific range. For this study we chose to analyze two strains with a significantly lower average ovariole number than *D. melanogaster* Oregon R (*OR*). The *India* (*Ind*) strain of *D. melanogaster* has an average of 24.4 ovarioles per female (taken as double the average value per ovary), while *D. sechellia* (*Ds*) has an average of 15.2 ovarioles per female, both of which are significantly lower than the *OR* average of 36.4 ovarioles per female (Table 1). *Ind* likely shared a last common ancestor with *OR* in Africa prior to human commensal dispersal in the Neolithic (Capy et al., 2004). *Ds* diverged from the lineage containing *D. melanogaster* approximately 5.4 million years ago (Tamura et al., 2004), and has evolved a single-niche specialization on the *Morinda citrifolia* fruit as its plant host in the Seychelles (Rkha et al., 1997). Given the relatively higher ovariole numbers observed in most other members of the *melanogaster* subgroup (Fig. 1), we therefore hypothesized that the reduction in ovariole number had occurred independently in the *Ind* and *Ds* lineages. To test this hypothesis, we performed an ancestral state reconstruction for ovariole number across the *Drosophila* family to generate a prediction for the ovariole number in the ancestor to *OR*, *Ind* and *Ds*. The maximum likelihood estimate for the average ovariole number of the ancestor to *OR*, *Ind*, and *Ds* is 31.7 ovarioles per female, with a 95% confidence interval of 25.2–38.3 ovarioles (Fig. 1 and Table S1). Average ovariole number per female in *OR* (36.4) is within this range, indicating that ovariole number in *OR* is not significantly different from the number hypothesized for its shared ancestor with *Ind* and *Ds*. However, average ovariole number in both *Ds* (15.2) and *Ind* (24.8) are below the ancestral range, indicating that ovariole number was independently reduced in both of these lineages. To address the possibility that *Ind* represents a segregating variant of the North American *D. melanogaster* range, we note that ovariole numbers in Indian populations (Rajpurohit et al., 2008) are, on average, smaller than those in North American populations (Capy et al., 1993). We also counted ovariole number in five additional *D. melanogaster* strains from North America, and found that their average ovariole numbers were always higher than those for *Ind* (Table 1). Taken together, these data show that ovariole number in *OR* is similar to the ancestral state of these three lineages, and reduced ovariole number convergently evolved in *Ind* and *Ds*.

TF cell number at the larval–pupal transition stage approximately equals adult ovariole number in Drosophila

Ovariole morphogenesis depends on the proliferation and differentiation of somatic gonad cells during early larval stages, and subsequent terminal filament (TF) formation during later larval stages (Fig. 2A). We previously showed that adult ovariole number between the cosmopolitan species *D. melanogaster* and *D. yakuba* is correlated with the number of a specific ovarian cell type, TF cells, at the LP stage (Sarikaya et al., 2012). Here we asked whether a difference in TF cell number also explained ovariole number differences in intraspecies and ecological specialist species comparisons. We found that in both *Ind* and *Ds*, TF number at the LP stage determines adult ovariole number (Fig. 2B). Previous studies had suggested that TF cell number per terminal filament or TF cell size might influence TF number and thus ovariole number (Hodin and Riddiford, 2000). We examined both of these parameters and found that neither was sufficient to account for ovariole number differences between the lineages (Fig. S1). Both TF number and TF cell number at the LP stage are thus robust predictors of ovariole number within and between *Drosophila* species, and also among *Drosophila* species that occupy varying ecological niches (Fig. 2C). We also tested the hypotheses that the TF cell number variation between these lineages was due to overall growth differences of the entire fly or of the ovary, or to differences in germ cell number. We found that neither hypothesis was supported. Neither germ cell number at any pre-LP stage of development (Figs. 3A and S2A) nor ovary size (Figs. 3B,C, and S2D and E) nor body size (Figs. 3D and S2B) was significantly correlated with adult ovariole number or TF cell number. The *Ds* ovary is significantly smaller than the *OR* ovary (Figs. 3B and C) and contains fewer TF cells

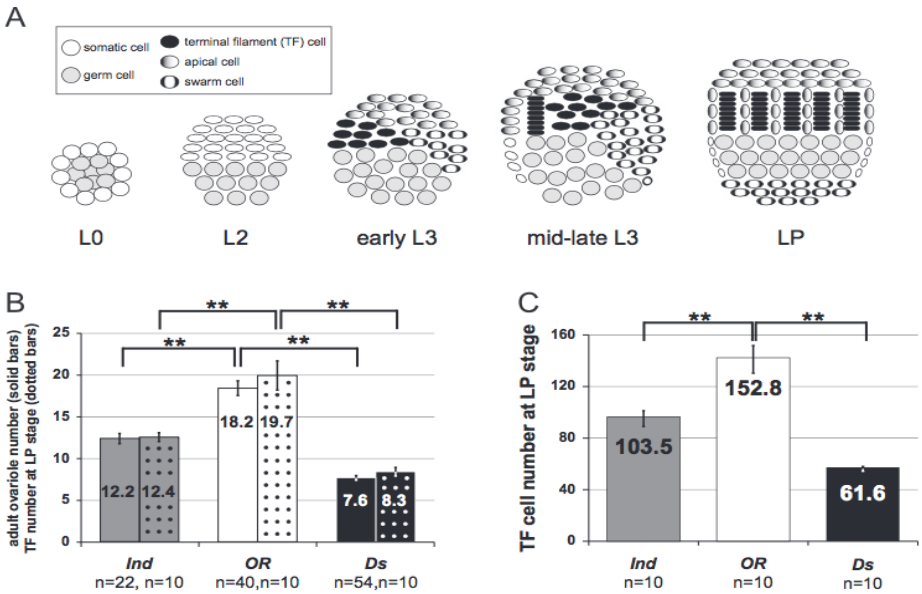

Fig. 2. Reduced ovariole number within and between *Drosophila* species is a result of reduced terminal filament cell number: (A) Schematic of ovary development and ovariole formation (see text for details). (B) Mean adult ovariole number (solid bars) and mean LP stage terminal filament (TF) number (stippled bars) in all three lineages. (C) Mean TF cell number at LP stage in all three lineages. In (B) and (C), n = number of ovaries analyzed (in (B), $n=x$, y are number of ovaries analyzed for ovariole number (x) and TF number measurements (y) respectively), error bars show 95% confidence interval, $^{**}p < 0.001$.

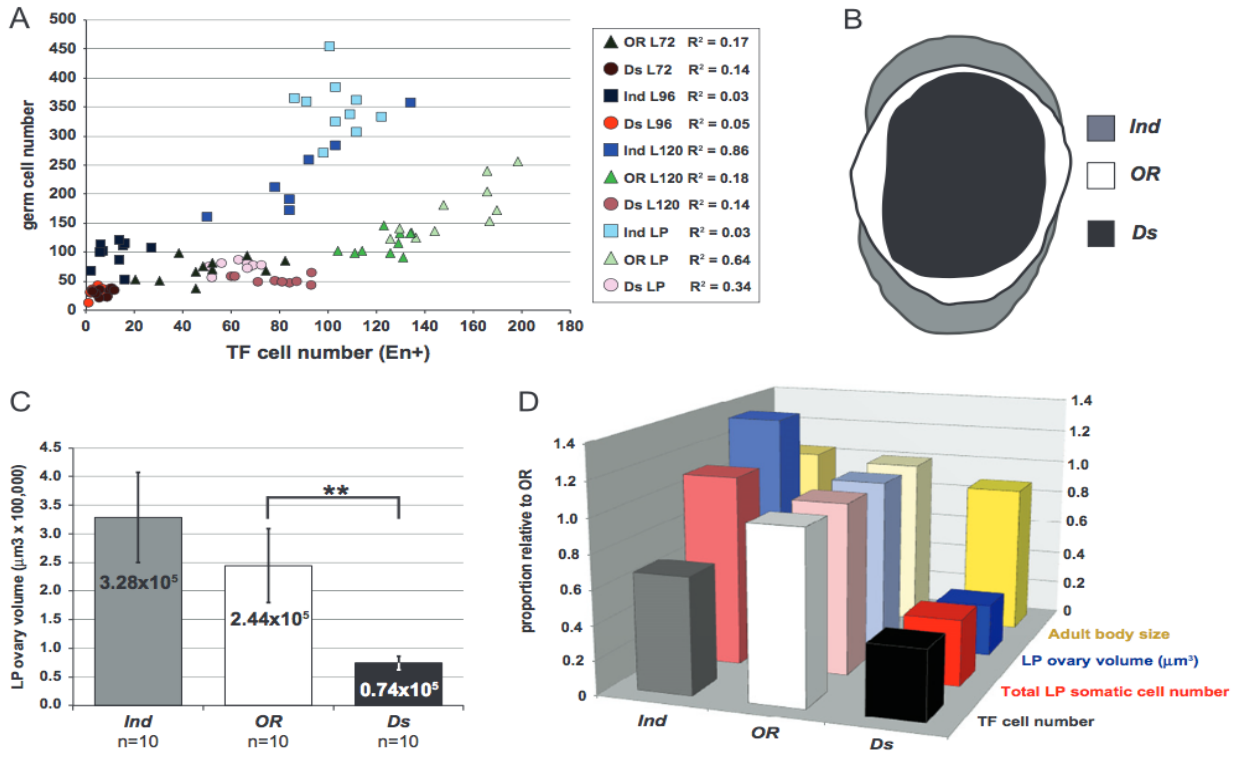

Fig. 3. Adult body size, germ cell number, and larval ovary size do not predict TFC number: (A) number of germ cells and TF cells from L0 to LP stage in *OR*, *Ind* and *Ds*. Each point shows germ cell and TF cell counts from a single individual. (B) Trace of optical cross-section through the widest point of an ovary of each lineage. (C) Mean larval ovary volumes for all three lineages. Error bars show 95% confidence interval, $^{**}p < 0.001$. (D) Relative proportions of adult body size (yellow shades) LP stage ovary volume (blue shades), total ovarian somatic cell number (red shades) and TF cell number (gray/white/black bars) for all three lineages. For each parameter, value is normalized to the corresponding *OR* value.

(Figs. 2C and S2D). However, the *Ind* ovary is slightly bigger than the *OR* ovary (Figs. 3B and C) yet has significantly fewer TF cells (Figs. 2C and S2D). This suggests that in *Drosophila*, specific mechanisms exist for precise control of TF cell number, leading to lineage-specific ovariole number.

A constant proportion of anterior somatic ovarian cells are specified as TF cells

At the LP stage, somatic cells of the ovary lie both anterior and posterior to germ cells (Fig. 2A). TF cells are derived exclusively from the anterior cell population. We asked if the three *Drosophila* lineages specified different proportions of TF cells from anterior cells. We found that the total number of anterior cells is different among lineages ($p < 0.05$), but across all three lineages, a similar proportion of anterior somatic cells differentiate into TF cells ($p > 0.05$; Fig. 4). This suggests that anterior somatic cell number is the key parameter that determines TF cell number. We therefore investigated the developmental origin of anterior cells, and whether decreased TF cell number in *Ind* and *Ds* compared to *OR* is a consequence of the same or different developmental mechanisms.

A reduced number of somatic gonad precursor cells established during embryogenesis leads to reduced TF cell number in Ds

Ovarian development begins during embryogenesis when a small number of somatic gonad precursor (SGP) cells are specified in the mesoderm of abdominal parasegments 10–12 (Boyle and DiNardo, 1995) (Fig. 2A). SGP cells undergo up to one mitotic

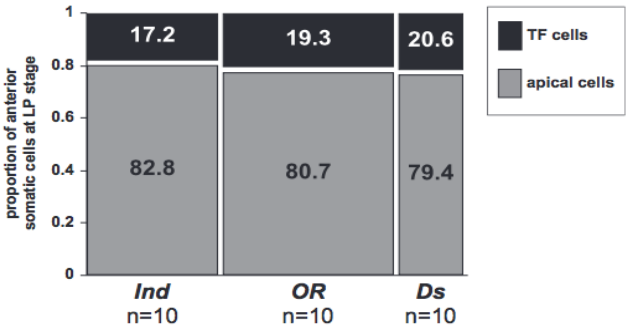

Fig. 4. Anterior somatic cell number, and not germ cell number, predicts TF cell number in *OR*, *Ind*, and *Ds*. Mosaic plots of proportions of the two anterior cell types, TF cells (black) and apical cells (gray) at LP stage in all three lineages. Bar width is proportional to total cell number in a given lineage.

division before hatching (L0), resulting in a small gonad primordium in the first larval instar. Somatic gonad cells proliferate and remain largely morphologically undifferentiated until later larval stages. During mid-third instar, a group of anterior somatic cells called "swarm" cells (Couderc et al., 2002) migrate laterally past the germ cell cluster towards the posterior of the ovary (Figs. 2A and S3). Once they are posterior to the germ cells, somatic cells differentiate to form the interfollicular stalk, basal stalk, and basal cells in later larval and pupal development (Couderc et al., 2002). A subset of the cells that remain anterior to the germ cells express

Engrailed and become TF cells (Bolívar et al., 2006; Godt and Laski, 1995). By the LP stage, anterior somatic ovary cells are thus divided into two cell populations: cells that express Engrailed (TF and cap cells), and those that do not (apical cells, which will migrate posteriorly between TF stacks to delineate individual ovarioles). We counted the number of gonadal cell types throughout ovary development in *OR*, *Ind*, and *Ds*. The number of SGP cells in *Ds* L0 larvae is significantly smaller than in *OR* ($p < 0.001$; Figs. 5A, A′ and S4A, A′), and the pool of somatic gonad cells remains comparatively smaller throughout development (Fig. S4A). Importantly, this difference is specific to the somatic gonad and does not reflect a reduction in primordial ovary size as a whole, as L0 germ cell number is similar across all three lineages at this stage (Figs. S4B, B′). As a result, *Ds* has a reduced number of all somatic cell types, including TF cells, at the LP stage (TF cells: $p < 0.001$; apical cells: $p < 0.001$; posterior cells (formerly swarm cells): $p < 0.001$; Figs. 5B, B′, C, C′. The same proportion of "swarm" cells migrate to the posterior in both *OR* and *Ds* ($p > 0.05$; Fig. 5C). Taken together, these data show that the developmental basis of evolutionary reduction in *Ds* ovariole number is primarily a change in the number of SGP cells initially established during embryogenesis.

Changes in ovarian morphogenesis during late larval stages lead to reduced TF cell number in Ind

In contrast to what we observed in *Ds*, SGP cell number is not significantly different between *Ind* and *OR* ($p = 0.95$; Figs. 5A, A′ and S4A, A′). Somatic cell proliferation rates between *Ind* and *OR* are similar (Fig. S4A), and both lineages reach similar numbers of total somatic cells by the LP stage ($p = 0.27$, Figs. 5B, B′). We therefore examined swarm cell migration and anterior/posterior somatic cell allocation in these ovaries. We found that significantly more swarm cells migrate to the posterior of the ovary in *Ind* than in *OR* ($p < 0.001$; Figs. 5C, C′). As a consequence, a significantly smaller proportion of cells are allocated to anterior cell fates in *Ind* than in *OR* ($p < 0.001$; Figs. 5C, C′). Because the same proportion of anterior cells become TF cells in these strains (Fig. 4B), we conclude that differences in swarm cell migration cause the observed reduction in *Ind* TF cell number relative to *OR*. These data indicate that *Ind* ovariole number reduction proceeds through different developmental mechanisms than those operating in *Ds*: rather than a difference in embryonic SGP cell establishment, in *Ind* descendants of the same initial number of SGP cells are allocated to specific cell fates in dramatically different ways. Notably, the variations in ovarian development occur at very different stages in *Ds* and *Ind*, but the final effect on TF cell number is nonetheless the same.

Loss of bab2 function in D. melanogaster reduces TF cell number by affecting ovarian morphogenesis during larval stages

Because these two developmental events occur at different developmental times and involve distinct cellular behaviors, we hypothesized that different genetic mechanisms could direct these developmental processes independently of one another. Quantitative genetics approaches to ovariole number variation have implicated different loci linked to interspecies (Coyne et al., 1991; Orgogozo et al., 2006) and intraspecies (Bergland et al., 2008; Wayne et al., 2001; Wayne and McIntyre, 2002) variation. However, few candidate genes have been suggested and none of the genes contained in these loci have yet been tested functionally for a role in ovariole number. We therefore revisited these data in light of our new developmental data on the differences between ovarian development in *Ind*, *Ds* and *OR*.

We first looked for candidate loci that might play a role in ovarian morphogenesis, specifically swarm cell migration. A QTL study examining ovariole number in recombinant inbred lines of

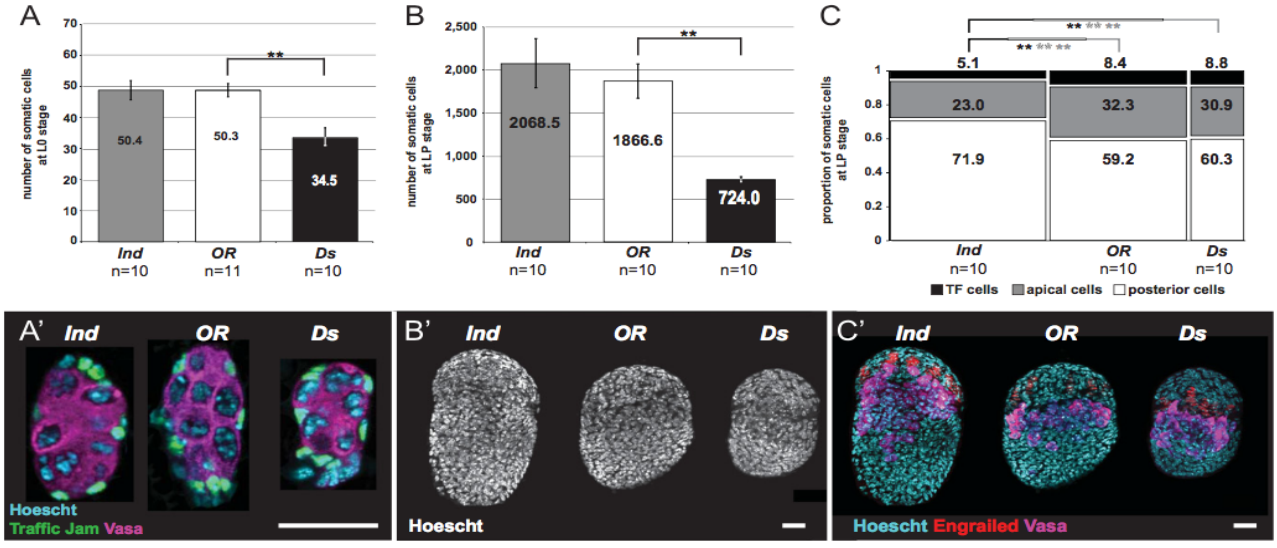

Fig. 5. Ovariole number reduction results from changes in the number of somatic gonad precursors in *Ds*, and from changes in somatic cell type segregation in the larval ovary in *Ind*: (A) mean somatic ovarian cell number at first larval instar (L0) 0–3 h after hatching (h AH) in all three lineages. (A′) Optical sections of L0 ovaries. (B) Mean total ovarian somatic cell number at LP stage in all three lineages. (B′) Maximum projections of optical sections of LP stage ovaries of *Ind*, *OR* and *Ds*. (C) Mosaic plots of the proportions of the three somatic cell types at LP stage (TF cells (black), apical cells (gray), and posterior cells (white)). Bar width is proportional to total cell number in a given lineage. Asterisk and bracket colors correspond to significance level for specific cell types (black: TF cells; gray: apical cells; white: posterior cells). (C′) Same images as in B′ but with cell populations distinguished by gene expression. TF cell values include a small proportion of cells that will become cap cells; cap cell number per TF is constant among all lineages observed (not shown). Intermingled cells (Li et al., 2003) are classified here as posterior cells; their number regulates germ cell number (Gilboa and Lehmann, 2006). Anterior is up in B′ and C′. All scale bars are 20 μm. $^{**}p < 0.001$. In (A), (B) error bars show 95% confidence interval.

D. melanogaster (strains distinct from those used in this study) identified a major effect locus that contains the *bric-á-brac* locus (Bergland et al., 2008), which encodes for the two genes *bab1* and *bab2* (Couderc et al., 2002). Both genes are expressed in the late larval ovary, are not expressed in the embryonic gonad, and play a role in ovarian morphogenesis (Godt and Laski, 1995; Sahut-Barnola et al., 1995). Because *bab2* is highly expressed in swarm cells at the time of their migration (Couderc et al., 2002), we hypothesized that specifically reducing *bab2* function in the somatic ovary might affect cell migration behavior and consequently TF cell number. We used the *bab:GAL4* driver (Cabrera et al., 2002), which is expressed in somatic cells of the larval ovary and most strongly in anterior somatic cells, to knock down *bab2* function in these cells via RNAi (Fig. 6A). *bab:GAL4 ≫ bab2^{RNAi}* did not alter the number of SGP cells specified relative to controls, *OR*, or *Ind* ($p=0.37$, 0.88, 0.85, respectively; Fig. 6B). However, we found that in *bab:GAL4 ≫ bab2^{RNAi}* ovaries, swarm cell migration was incomplete by the LP stage (Fig. 6A) and the number of swarm cells was significantly higher than controls ($p<0.001$; Fig. 6C) of swarm cells were affected. We counted all cell types in these ovaries at the LP stage to quantify the effects on anterior/posterior somatic cell allocation, and found that *bab2* knockdown resulted in a significantly greater proportion of posterior cells at the expense of anterior cells ($p<0.001$; Fig. 6C). Interestingly, average anterior/posterior proportions were nearly identical to those in *Ind* ($p<0.001$; Figs. 5C and 6C). The proportion of anterior cells that became TF cells was similar to all three wild type lineages and *bab2^{RNAi};+* controls ($p=0.13$ (*Ind*), 0.40 (*OR*), 0.76 (*Ds*), 0.02, respectively; Fig. S5A).

Because *bab* also plays a role in the process of TF cell stacking to form TFs (Godt and Laski, 1995), *bab:GAL4 ≫ bab2^{RNAi}* ovaries ultimately fail to make normal TFs or ovarioles. However, as a consequence of reduced anterior cell number, TF cell number was reduced in *bab:GAL4 ≫ bab2^{RNAi}* ovaries compared to controls (Fig. S5B), suggesting that TF number and adult ovariole number would also be reduced in these females. These results show that changes in *bab2* function can influence TF cell number by affecting swarm cell migration, thereby altering the anterior/posterior proportioning of somatic ovary cells. Importantly, body size was unchanged in *bab:GAL4 ≫ bab2^{RNAi}* females compared to controls ($p=0.64$; Fig. S5C), demonstrating that ovariole number can be changed independently of body size. These phenotypes mimic the critical developmental differences during larval development that underlie ovariole number differences between *OR* and *Ind*, while leaving SGP cell establishment in embryogenesis unaltered.

Loss of InR function in D. melanogaster reduces TF cell number by affecting SGP cell establishment

We next examined previous QTL analyses for genes that might affect TF cell number by affecting SGP cell number. The *Drosophila Insulin receptor* (*InR*) gene emerged as a top candidate for investigation. *InR* is contained within a large-effect locus linked to ovariole number difference between *D. simulans* and *D. sechellia* (Orgogozo et al., 2006). The *D. sechellia* strain used in that QTL study (DSSC #14021-0248.07) was collected in the same year (1980) and at the same location (Cousin Island, Seychelles) as the strain used in the present study (DSSC #14021-0248.25), which is the strain used to construct the BAC library for genome sequencing (Drosophila 12 Genomes Consortium, 2007). *InR* is the single insulin-like peptide receptor in *Drosophila* that mediates the insulin signaling pathway (Petruzzelli et al., 1986), a major regulator of cell proliferation and body size in animals (Goberdhan and Wilson, 2003). Reduced insulin signaling in *Drosophila* leads to reduced body size as a consequence of reductions in both cell number and cell size (Böhni et al., 1999; Chen et al., 1996; Shingleton et al., 2005), but patterning and morphogenesis programs remain intact. Moreover, loss-of-function mutants in the InR substrate *chico* have reduced adult ovariole number (Richard et al., 2005; Tu and Tatar, 2003).

We hypothesized that flies with reduced insulin signaling activity would have a lower adult ovariole number due to a reduced number of SGP cells specified during embryogenesis, thereby reducing TF cell number. We confirmed that *InR$^{E19/GC25}$* loss-of-function trans-heterozygotes contain significantly fewer TFs ($p<0.001$; Fig. 6A) and TF cells ($p<0.001$; Fig. S5B) at the LP stage compared to heterozygous controls (either *InR^{E19}/+* or *InR^{GC25}/+*) and *OR*. Consistent with our hypothesis, we found that SGP cell number at the L0 stage was significantly smaller in *InR$^{E19/GC25}$* compared to *OR* (Fig. 6B). In heterozygotes both *InR* alleles had significantly reduced SGP cell number ($p<0.005$; Fig. 6B) and TF cell size ($p<0.001$; Fig. S5D), but TF cell number ($p=0.31$; Fig. S5B) and TF number ($p=0.72$; Fig. 6A) were not significantly different from *OR*. TF cell size was also reduced in *InR$^{E19/GC25}$* trans-heterozygotes compared with controls and *OR* (Fig. S5D), but as observed for lineage-specific cell size differences (Fig. S1B), this did not account for the reduction in TF number (Fig. 6A). Swarm cell migration was not affected, so that the anterior/posterior proportions of somatic cells were similar between *InR$^{E19/GC25}$* and heterozygote controls (36.6% anterior/63.4% posterior vs. 40.6% anterior/59.4% posterior respectively, $p<0.05$)

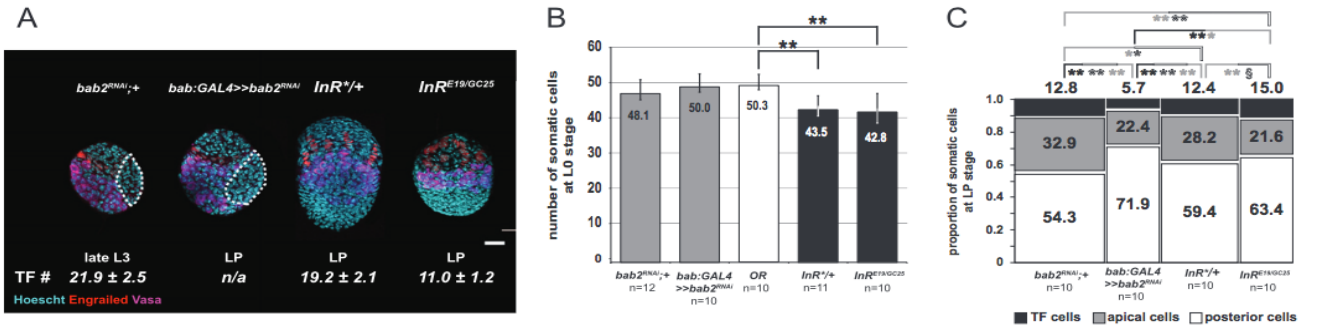

Fig. 6. Distinct genetic mechanisms regulate ovariole number by altering SGP cell number changes or cell type allocation: (A) ovaries of *bab2^{RNAi};+*, *bab:GAL4»bab2^{RNAi}*, *InR^{E19}* or *InR^{GC25}/TM3*, and *InR^{E19}/InR^{GC25}* at LP stage, except for *bab2^{RNAi};+*, shown at late-third instar to visualize swarm cell migration. All images are maximum projections of optical sections, anterior is up, scale bar=20 μm. (B) Mean SGP cell number at L0 for *bab2^{RNAi};+*, *bab:GAL4 ≫ bab2^{RNAi}*, *InR^{E19}* or *InR^{GC25}/TM3*, *InR^{E19}/InR^{GC25}* and *OR* ovaries. Error bars show 95% confidence interval. (C) Mosaic plots of proportions of somatic cell types at LP stage in genetic backgrounds shown in (A). Bar width is proportional to total cell number in a given lineage. Asterisk and bracket colors correspond to significance level for specific cell types (black: TF cells; gray: apical cells; white: posterior cells).

but significantly different compared to *Ind* (28.1% anterior/71.9% posterior, $p < 0.005$) and *bab:GAL4»bab2*^{RNAi} (28.2% anterior/71.9% posterior, $p < 0.01$; Figs. 5C and 6C). We did observe that in *InR*$^{E19/GC25}$ the proportion of anterior cells that differentiated into TF cells was elevated compared to controls and all three wild type lineages (controls: $p < 0.01$; *OR*, *Ind*: $p < 0.001$; *Ds*: $p < 0.005$; Fig. 6C), accounting for the apparently significant difference ($p < 0.05$) in the overall anterior/posterior proportioning of cells in *InR*$^{E19/GC25}$ compared to heterozygote controls. TF cell number per TF stack was also increased relative to controls ($p < 0.001$; Fig. S5E). Consistent with recent reports on a role for hormonal signaling in germ line stem cell niche formation (Gancz et al., 2011), these observations suggest that in addition to controlling cell proliferation, insulin signaling may also play a role in TF cell fate specification and morphogenesis. Nevertheless, these changes in TF cell allocation and morphogenesis do not compensate for the reduced number of anterior somatic cells in *InR*$^{E19/GC25}$ ovaries (Fig. S5A), so that the ultimate result is specification of fewer TF cells and fewer TFs (Figs. 6A and S5B). Therefore, reduced insulin signaling lowers ovariole number principally through reducing the number of SGP cells established during embryogenesis, rather than through changes in larval ovarian development. In this way, reduced insulin signaling phenocopies the essential developmental differences that cause ovariole number difference between *OR* and *Ds*.

Discussion

Convergent evolution of reduced ovariole number by distinct developmental mechanisms

We have shown that independent instances of evolutionary reduction in ovariole number can result from alterations in different developmental processes (Fig. 7). In *Ds*, a smaller somatic gonad primordium than that of *OR* is established by hatching, although L0 germ cell number and all other later ovariole developmental processes that we examined are similar between the two species. *Ds* therefore has fewer of all somatic cell types of the ovary, including TF cells, and as a consequence forms fewer TFs and fewer ovarioles. In contrast, the L0 gonad of *Ind* is initially the same size as that of *OR*. During larval development, a smaller proportion of the somatic gonad cells in *Ind* are allocated to TF fate due to differences in somatic cell migration within the gonad. As a result, fewer TFs and fewer ovarioles are formed.

Different genetic mechanisms independently regulate these different developmental processes in *D. melanogaster*. Reduction of insulin signaling pathway activity results in fewer L0 gonad primordium cells and fewer TFs, but does not affect migration behaviors later in ovarian development. In contrast, loss of *bab2* function in somatic gonad cells alters their migration behaviors, but does not affect L0 gonad size. Taken together with QTL linkage of *InR* and *bab* to ovariole number variation, this suggests that changes in distinct genetic pathways may underlie modular evolution of ovariole number in *Drosophila*, which could contribute to the high evolutionary lability of this trait.

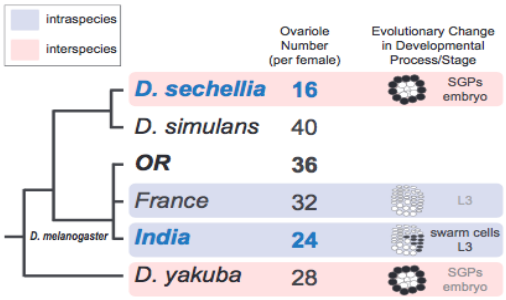

Fig. 7. Different developmental mechanisms underlie ovariole number evolution. Lineages studied in detail in this report are shown in bold. Independent reductions in ovariole number (blue text) evolved in the *melanogaster* subgroup via distinct developmental mechanisms that affect different cell types and developmental stages.

Our results suggest that previous QTL analyses may have identified non-overlapping loci that contribute to ovariole number variation in *Drosophila* because the lineages under comparison diverged in different developmental mechanisms that underlie ovariole number determination. One interesting question is whether or not patterns of intraspecies versus interspecies ovariole number variation exist in *Drosophila*. We note the interesting agreement between the developmental differences we observe between *OR*, *Ds*, and *Ind*, and their candidate genetic bases suggested by previous inter- and intraspecies QTL analyses and the present analyses. L0 somatic cell specification, the principal developmental process accounting for ovariole number difference between *OR* and *Ds*, is regulated by *InR*, which was a major QTL locus from an interspecies analysis (Orgogozo et al., 2006). Larval ovary morphogenesis, differences in which underlie the ovariole number difference between *OR* and *Ind*, is regulated by *bab2*, and the *bab* locus including *bab2* was a major QTL locus from an intraspecies analysis (Bergland et al., 2008). Our preliminary analysis of additional lineages suggests that the genetic basis for change in this trait may show some consistency between and within *Drosophila* species. We have found that different developmental processes produce the TF cell number differences underlying ovariole number differences in *D. yakuba* (Sarikaya et al., 2012) and in the *D. melanogaster France* strain, both of which have fewer ovarioles than *OR* (Table 1). The size of the L0 ovarian primordium is significantly smaller than *OR* in *D. yakuba* (Fig. S6; $p < 0.001$), as in *Ds*. This suggests that ovarian primordium size differences contribute to ovariole number variation between species, and that SGP specification may be a species-specific trait. However, *France* L0 primordium size is similar to that of *OR* (Fig. S6; $p = 0.025$), as we reported here for *Ind*, indicating that changes in larval ovary development are responsible for reducing ovariole number in *France* compared to *OR*. Thus among these *D. melanogaster* strains, larval developmental processes, rather than embryonic ones, are a major source of variation in ovariole number.

Multiple developmental mechanisms affecting ovariole number may provide different opportunities for evolutionary change

Because ovariole number is determined by TF number at the larval–pupal transition, it is a complex trait that requires multiple developmental processes: embryonic establishment of the somatic gonad, proliferation during larval life, migration of a specific proportion of somatic gonad cells, differentiation of some anterior somatic cells into TF cells, and finally TF cell stacking to form TFs (Fig. 2A). Each of these steps is directed by very different mechanisms, each of which could conceivably be the target of evolutionary change. We propose that evolutionary change in ovariole number may be particularly likely to proceed via a diverse set of developmental and possibly genetic mechanisms because of its cell type complexity. The ovary is composed of multiple cell types that each follows an individual developmental program, and yet must be integrated to form a functional organ. The *Drosophila* sex combs are a similar example of a complex multicellular structure whose convergent evolution can proceed through multiple different developmental mechanisms (Tanaka

et al., 2009). Interestingly, both ovariole number and sex comb morphology show high evolutionary lability, perhaps indicating that complex traits provide a broad "evolutionary change landscape" that allows for rapid diversification via multiple routes.

In many cases where convergent morphological traits evolve via changes in the same genetic mechanisms, these morphological traits are terminal differentiation aspects of a single type of somatic cells. For example, the degree of expression of a pigment synthesis pathway (Protas et al., 2006), or the accumulation of cortical actin that determines the formation of an epidermal bristle (Sucena et al., 2003), are likely to be processes that are cell-autonomous and do not require significant coordination with other cell types. The developmental processes operating prior to this differentiation will surely require cooperation of multiple cell types, but a single cell expresses pigment or develops a bristle autonomously. We speculate that this developmental feature may facilitate convergent evolution through mutations in the same developmental pathway. In contrast, ovarioles are multicellular rather than cell-autonomous structures, and as such evolution may have many "opportunities" to change this and other complex traits through multiple genes directing several distinct processes at different times in development.

Towards the genetic basis of ovariole number variation

By characterizing the developmental basis of phenotypic differences in a rapidly evolving trait, we were able to suggest novel specific hypotheses regarding particular candidate genes within previously identified QTL regions associated with ovariole number variation. This suggests that for many complex traits, a better understanding of the underlying developmental processes can be a fruitful way to interpret the results of QTL analyses and enhance their utilities for functional studies, by identifying relevant candidates and excluding others. Indeed, such an integrative approach has previously succeeded in identifying genetic loci of evolutionary change in pelvic reduction between lineages of threespine sticklebacks (Shapiro et al., 2004). Our functional experiments in *D. melanogaster* revealed two different genes that can regulate ovariole number in different ways. Loss of function of *InR* and *bab* not only reduce ovariole number in *D. melanogaster*, but do so by affecting the same developmental mechanisms that reduce ovariole number in *Ds* and *Ind*, respectively. The data shown here provide, to our knowledge, the first functional test of specific candidate genes within QTL linked to ovariole number variation (Bergland et al., 2008; Orgogozo et al., 2006; Wayne et al., 2001), and suggest these genes as potential causal loci for change in this trait. We speculate that evolution at the *bab* locus may underlie reduced ovariole number in *Ind*, while changes of *InR* function could be responsible for the evolution of reduced ovariole number in *Ds*. Evolutionary changes at the *bab* locus have been previously implicated in the evolution of adult abdominal pigmentation and trichome patterns in *Drosophila* species (Gompel and Carroll, 2003; Kopp et al., 2000), but the nature of the selective pressures acting on this locus are poorly understood. *bab* plays multiple roles in development, including TF formation (Godt and Laski, 1995; Sahut-Barnola et al., 1995), leg development (Couderc et al., 2002), and a role in somatic ovary cell migration that we describe here for the first time. The role of *bab* in ovariole number is likely to have a direct impact on fertility and therefore fitness. It may be that ectodermal patterning variation resulting from *bab* modification is a secondary effect of selection on *bab's* role in ovarian morphogenesis, or vice versa.

With respect to *InR*, several lines of evidence suggest that evolutionary change in insulin signaling (INS) genes plays an important role in ovariole number variation. First, consistent with its QTL linkage to interspecies ovariole number variation (Orgogozo et al., 2006), the specific developmental processes affected by *InR* loss of function correspond to those that vary between *Drosophila* species. Second, clinal variation in *InR* alleles has been observed in natural populations of *D. melanogaster* (Paaby et al., 2010), and ovariole number also exhibits clinal variation (Boulétreau-Merle et al., 1992; Collinge et al., 2006; David and Bocquet, 1975; Delpuech et al., 1995; Gibert et al., 2004; Paaby et al., 2010; Schmidt et al., 2005; Wayne et al., 2005). Third, analysis of clinal alleles reveals evidence of positive selection at the *InR* locus (Paaby et al., 2010). Finally, different organs are known to respond differently to changes in INS in *Drosophila* (Shingleton et al., 2005; Tang et al., 2011), providing mechanisms for putative organ-specific responses to changes in a global hormonal pathway, and consistent with the altered correlations between ovariole number and overall body size between species (Bergland et al., 2008; Hodin and Riddiford, 2000, this report).

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2012.09.014.

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