

# Soma-dependent modulations contribute to divergence of *rhomboid* expression during evolution of *Drosophila* eggshell morphology

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Patterning of the respiratory dorsal appendages (DAs) on the *Drosophila melanogaster* eggshell is tightly regulated by epidermal growth factor receptor (EGFR) signaling. Variation in the DA number is observed among *Drosophila* species; *D. melanogaster* has two DAs and *D. virilis* has four. Diversification in the expression pattern of *rhomboid* (*rho*), which activates EGFR signaling in somatic follicle cells, could cause the evolutionary divergence of DA numbers. Here we identified a cis-regulatory element of *D. virilis rho*. A comparison with *D. melanogaster rho* enhancer and activity studies in homologous and heterologous species suggested that these *rho* enhancers did not functionally diverge significantly during the evolution of these species. Experiments using chimeric eggs composed of a *D. virilis* oocyte and *D. melanogaster* follicle cells showed the evolution of DA number was not attributable to germline Gurken (Grk) signaling, but to divergence in events downstream of Grk signaling affecting the *rho* enhancer activity in somatic follicle cells. We found that a transcription factor, Mirror, which activates *rho*, could be one of these downstream factors. Thus, evolution of the trans-regulatory environment that controls *rho* expression in somatic follicle cells could be a major contributor to the evolutionary changes in DA number.

**KEY WORDS:** *Drosophila melanogaster*, *Drosophila virilis*, *rhomboid*, *mirror*, *gurken*, *Broad-Complex*, EGFR signaling, cis-regulatory element, trans-regulatory landscape, Evolution, Eggshell, Dorsal appendage

## INTRODUCTION

Evolutionary changes in gene regulation are a major cause of the diversification of morphological traits in animals (Carroll et al., 2001; Davidson, 2001; Simpson, 2002). Although differences in the expression patterns of genes with instructive roles in morphogenesis have been demonstrated in various species (Averof and Patel, 1997; Gompel and Carroll, 2003; Shapiro et al., 2004; Simpson et al., 1999; Stern, 1998), few studies have addressed the molecular mechanisms underlying the diversification of these gene expression patterns (Belting et al., 1998; Gompel et al., 2005; Wittkopp et al., 2002). The dorsal appendages (DAs) are specialized respiratory structures on the drosophilid eggshell (Hinton, 1969; Spradling, 1993a). The mechanisms of their morphogenesis have been extensively studied in *Drosophila melanogaster*, whose eggshell has two DAs (Fig. 1B) (Berg, 2005). There is remarkable diversity in the DA morphology among species, including their size, shape and number, although these characteristics are stereotyped within each species (Kambysellis and Craddock, 1997; Patterson and Stone, 1952; Throckmorton, 1962). Thus, comparative studies of gene expression patterns during DA formation are useful for understanding the mechanisms underlying the evolutionary diversification of gene expression (Barkai and Shilo, 2002; Derheimer et al., 2004; James and Berg, 2003; Nakamura and Matsuno, 2003; Peri et al., 1999; Perrimon and Duffy, 1998).

The egg chamber of *Drosophila* consists of a single oocyte and 15 nurse cells surrounded by a layer of somatic follicle cells (Margolis and Spradling, 1995; Spradling, 1993a). In *D. melanogaster*, the DA primordia arise from a subset of follicle cells that are specified by epidermal growth factor receptor (EGFR) signaling (Nilson and Schupbach, 1999; Wasserman and Freeman, 1998). Gurken (Grk) is a transforming growth factor- $\alpha$ -like protein and an oocyte-specific ligand for EGFR that localizes to the dorsal-anterior end of the oocyte and is presented to the overlying follicle cells (Neuman-Silberberg and Schupbach, 1993; Nilson and Schupbach, 1999; Wasserman and Freeman, 1998). Grk induces the expression of *rhomboid* (*rho*), which encodes a serine protease, in a single population of dorsal anterior follicle cells (Lee et al., 2001; Ruohola-Baker et al., 1993; Urban et al., 2001), and the Decapentaplegic signaling pathway helps limit the expression of *rho* in these cells (Peri and Roth, 2000). In these follicle cells, Rho processes Spitz, a transmembrane ligand for EGFR, to a secreted and active form, which in turn amplifies EGFR signaling in the follicle cells (Sapir et al., 1998; Schweitzer et al., 1995; Wasserman and Freeman, 1998). The high EGFR signaling activity triggers the expression of *argos* at the dorsal anterior midline and subsequently establishes a negative feedback loop, resolving the single peak of EGFR signaling into twin peaks (Wasserman and Freeman, 1998). Consequently, a single DA primordium is formed at each region of peak EGFR signaling activity (Wasserman and Freeman, 1998).

*D. virilis*, a species that diverged from *D. melanogaster* 40-60 million years ago, has four DAs (Fig. 1A,C) (Powell, 1997). We previously showed that *rho* is expressed differently between *D. melanogaster* and *D. virilis* (Fig. 1D,E). At stage 10A, *rho* is expressed in *D. melanogaster* in a dorsal anterior saddle-shaped zone that includes the midline (Fig. 1D), but in *D. virilis* there are two dorsal-lateral domains (Fig. 1E). At stage 10B, *rho* expression is refined into two L-shaped stripes in *D. melanogaster* (Fig. 1D);

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the pattern in *D. virilis* becomes a V-shaped stripe with its apex missing (Fig. 1E). Finally, the *rho* expression is maintained in two regions of follicle cells where the two DAs will form in *D. melanogaster* at stage 12 (Fig. 1D), whereas in *D. virilis*, *rho* expression is restricted to four domains corresponding to the positions of the future DAs (Fig. 1E). Thus, changes in the regulation of *rho* expression could be responsible for the divergence in DA number (Nakamura and Matsuno, 2003). In this study, we investigated the mechanisms by which *rho* expression diverged during the evolution of *D. melanogaster* and *D. virilis*. Our results suggest that divergence of the trans-acting landscape regulating the *rho* expression probably had a crucial role in the evolution of different DA numbers in these two species.

## MATERIALS AND METHODS

### *Drosophila* strains

The *Drosophila* strains used as wild type were: *D. melanogaster*, Canton-S; *D. virilis*, stocks #15010-1051.0 and #15010-1051.87 (Tucson *Drosophila* Stock Center, <http://stockcenter.arl.arizona.edu>). The *D. melanogaster* *yw*<sup>7-5</sup> and *D. virilis* *w* (stock #15010-1051.53, *white*<sup>50112</sup>) mutant strains were used as the *D. melanogaster* and *D. virilis* transformation hosts, respectively. The *D. melanogaster* EGFP-*Vasa* strain (Sano et al., 2002) was used as a pole cell transplantation host.

### Immunohistochemistry

Immunofluorescent staining of ovaries was performed as described (James and Berg, 2003), except that Block Ace (Dainippon Pharmaceutical) was used as a blocking reagent. The following primary antibodies were used: rabbit anti-green fluorescent protein (GFP) (1:1000, MBL), mouse anti- $\beta$ -galactosidase (Gal) (1:500, Promega) and rabbit anti-Broad-Complex (BR-C) core (1:2000) (Dequier et al., 2001). Alexa Fluor-488-conjugated anti-rabbit immunoglobulin G (IgG) (1:400, Molecular Probes) and Cy3-conjugated anti-mouse IgG (1:400, Rockland) were used as secondary antibodies. Immunofluorescent staining of embryos was performed as described (Hayashi et al., 2004). Embryos were stained with rabbit anti-*Vasa* (1:500) and mouse anti-GFP (1:500, Wako Pure Chemicals) antibodies, and then treated with Alexa Fluor-568-conjugated anti-rabbit IgG (1:500, Molecular Probes) and Alexa Fluor-488-conjugated anti-mouse IgG (1:500, Molecular Probes).

### In situ hybridization

The RNA probes were labeled with digoxigenin (Roche), and in situ hybridization was performed as described (Wasserman and Freeman, 1998).

### Cloning of *mirr* (*mirr*)

Partial DNA fragments of *mirr* for in situ hybridization were amplified using genomic DNAs from Canton-S and *D. virilis* (#15010-1051.87) as template DNAs by PCR. The primers were: *Mirr\_FW*, 5'-GATATGATGACCGACC-3' and *Mirr\_RV*, 5'-CCTATAAGCTCTGATTGC-3'.

### Cloning of the *D. virilis rho* 5' regulatory DNA

The DNA probe of the *D. virilis rho* cDNA was labeled with digoxigenin (DIG DNA labeling Mix, Roche) by random-primed labeling (Nakamura and Matsuno, 2003). A *D. virilis* phage genomic library (gift of T. C. Kaufman, Indiana University, Bloomington, IN) was screened with this probe using standard conditions (Sambrook and Russel, 2001). Twenty-one overlapping phage clones that hybridized with the *D. virilis rho* probe were isolated. The three largest genomic fragments isolated from these phage clones were digested with *SalI* and subcloned into the *SalI* site of pBluescript KS(-). These subclones were sequenced, aligned and ligated. Finally, a genomic DNA fragment 12-kb upstream of the start site of the *D. virilis rho* cDNA was obtained (GenBank accession number AB278158).

### Transgene construction for *rho* enhancer analysis

A genomic fragment covering the 2.2-kb upstream region of the *D. melanogaster rho* gene was generated by PCR using the *D. melanogaster* genomic P1 clone DS02734 (GenBank accession number AC004343) as a template (Kimmerly et al., 1996), and subcloned into the *Bam*HI site of

pCaSpeR-NLSlacZ (gift of C. Thummel, Howard Hughes Medical Institute, University of Utah, Salt Lake City, UT). The primers were: *rho*2.2-FW, 5'-CGGGATCCCCGCAAGCTTTTCTCTGCTC-3' and *rho*2.2-RV, 5'-CGGGATCCCCGTTCTCTGCTTGCACCCAC-3. (Restriction enzyme cloning sites are underlined.)

The 2.2-kb upstream fragment of the *D. melanogaster rho* gene and fragments containing the 4.2- and 12-kb upstream regions of the *D. virilis rho* gene were subcloned into the *Bam*HI and *Kpn*I/*Bam*HI sites of pSL[hsp27mp-NLS-EGFP] (T.N., unpublished), respectively. The resulting constructs were digested with *Asc*I, and each insert was cloned into the *Asc*I site of pBac[3xP3-DsRedaf].

### Transformation of *D. melanogaster* and *D. virilis*

Germline transformation of the P-element CaSpeR vectors was performed according to a standard protocol (Spradling, 1993b). For transformation with the *piggyBac* vector, *piggyBac* constructs were co-injected into the eggs of *D. melanogaster yw* and *D. virilis w* with phsp-pBac, and flies were screened for 3xP3-DsRed expression under a fluorescence stereoscopic microscope (gift of E. A. Wimmer, Georg August University, Göttingen, Germany). For each transgene, at least three independent insertions were isolated and characterized.

### Pole cell transplantation

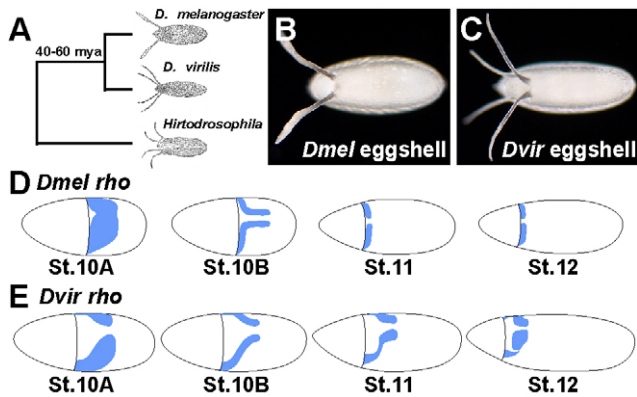
Pole cell transplantation was performed as described (Kobayashi et al., 1996), with the following modifications. As a marker for the *D. melanogaster* germline, we used an EGFP-*vasa* construct that expresses GFP specifically and continuously in the germline throughout the life cycle (Sano et al., 2002). We transplanted pole cells from *D. virilis* (#15010-1051.0) embryos [200-250 minutes after egg laying (AEL) (25°C)] into *D. melanogaster* embryos [100-150 minutes AEL (25°C)] carrying the EGFP-*vasa*. The donor embryos were at the cellular blastodermal stage.

## RESULTS

### Identification of the *D. virilis rho* enhancer responsible for *rho* expression in the DA-forming follicle cells

In *D. melanogaster*, a region of the *rho* gene 2.2-kb upstream of its transcriptional start site contains a cis-regulatory element that is responsible for its expression during oogenesis (Dorman et al., 2004; Ip et al., 1992; Sapir et al., 1998; Ward and Berg, 2005). This *rho* enhancer, termed Dmel rho<sup>2.2</sup>, is sufficient to drive the expression of a reporter gene in the developing follicle cells (Dorman et al., 2004; Sapir et al., 1998; Ward and Berg, 2005). At stage 10B, Dmel rho<sup>2.2</sup> is activated in L-shaped stripes on either side of the midline (Dorman et al., 2004; Sapir et al., 1998; Ward and Berg, 2005). This expression pattern resembles that of endogenous *rho*, which is essential for DA formation, at the same stage (Fig. 1D and Fig. 3C) (Dorman et al., 2004; Sapir et al., 1998).

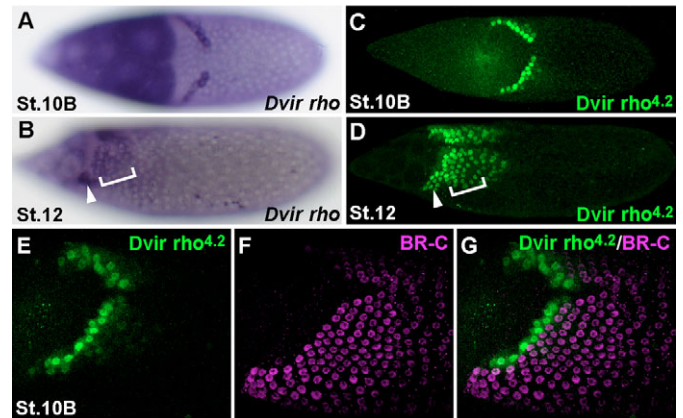
We speculated that a similar cis-regulatory element should exist in the *D. virilis rho* gene. Therefore, we isolated a 12-kb genomic region upstream of the *D. virilis rho* cDNA start site (GenBank accession number AB278158). To test whether the 12-kb fragment or a 4.2-kb upstream fragment derived from it contained a regulatory sequence that governs *rho* expression in the follicle cells, we analyzed the activity of these fragments in *D. virilis* using *piggyBac*-mediated transgenesis (Handler, 2002). When placed upstream of a GFP reporter gene, the 12- and 4.2-kb upstream fragments drove reporter expression in the follicle cells of *D. virilis* during DA formation in essentially the same manner (Fig. 2 and data not shown). Therefore, we used the 4.2-kb fragment in the following studies, because a shorter fragment is preferable for generating transgenic lines efficiently in *D. virilis* and *D. melanogaster*. At stage 10B, Dvir rho<sup>4.2</sup> drove reporter expression in a pattern identical to the distribution of endogenous *D. virilis rho* mRNA (Fig. 2A,C). At stage 12, Dvir rho<sup>4.2</sup> also drove GFP expression in the four



**Fig. 1. Evolutionary divergence of eggshell morphology and *rho* expression pattern between *D. melanogaster* and *D. virilis*.** (A) The egg of *D. virilis* has four DAs, an ancestral trait, as found in the *Hirtodrosophila* outgroup of the genus *Drosophila*. The two DAs found in *D. melanogaster* is a more recently evolved trait. *D. virilis* and *D. melanogaster* diverged 40–60 million years ago (mya). (B,C) The eggshells of *D. melanogaster* (B) and *D. virilis* (C) are shown with anterior to the left (dorsal view). (D,E) Schematic comparison of the *rho* expression patterns between *D. melanogaster* and *D. virilis* during oogenesis. Dorsal lateral views of stage 10A- to 12-egg chambers are shown with *rho* expression (blue). Anterior is to the left. (D) *D. melanogaster rho* was expressed in a saddle-shaped zone at the dorsal anterior at stage 10A. This expression was refined into an L-shaped stripe on either side of the dorsal midline at stage 10B. Expression was then restricted to the anterior row lacking the midline region at stage 11, as it continued until stage 12. (E) *D. virilis rho* was expressed in two domains at the dorsal-lateral sides at stage 10A. No expression occurred in the midline, unlike in *D. melanogaster*. The expression was repressed at the posterior region and refined into a V-shaped stripe missing its apex at stage 10B. Expression was upregulated at the front and rear of each stripe, and expression in the rear moved anteriorly at stage 11. Subsequently, the expression was divided into four domains at stage 12, corresponding to the positions of the four DAs.

domains that subsequently form the four DAs (Fig. 2D); this pattern was also similar to that of endogenous *rho* at the same stage (Fig. 2B). We concluded that *Dvir rho*<sup>4.2</sup> consists of an enhancer orthologous to *Dmel rho*<sup>2.2</sup>.

Broad-Complex (BR-C) is expressed in the presumptive DA-forming cells (Deng and Bownes, 1997; Tzolovsky et al., 1999). The juxtaposition of *rho*-expressing and BR-C-expressing cells is required for DA formation in *D. melanogaster* (Ward and Berg, 2005). In cells expressing *rho*, Spitz is converted into an active and secreted ligand for EGFR (Lee et al., 2001; Urban et al., 2001), so that EGFR signaling is activated and triggers the expression of BR-C in adjacent cells (Schweitzer et al., 1995; Ward and Berg, 2005). In *D. virilis* and *D. melanogaster*, the *rho* and BR-C expression domains are mutually exclusive at stage 10B (Nakamura and Matsuno, 2003), indicating that the genetic cascade involving *rho* and BR-C is conserved between these species. To test whether *Dvir rho*<sup>4.2</sup> carries sufficient information to restrict its activation anterior to the BR-C-expressing domain, we analyzed the expression of *Dvir rho*<sup>4.2</sup>-GFP and BR-C in *D. virilis*. *Dvir rho*<sup>4.2</sup> was activated in a single row of cells anterior and adjacent to BR-C-expressing cells (Fig. 2E–G). These results suggested that *Dvir rho*<sup>4.2</sup> contains sufficient information for comparative studies with *Dmel rho*<sup>2.2</sup> in these two species.



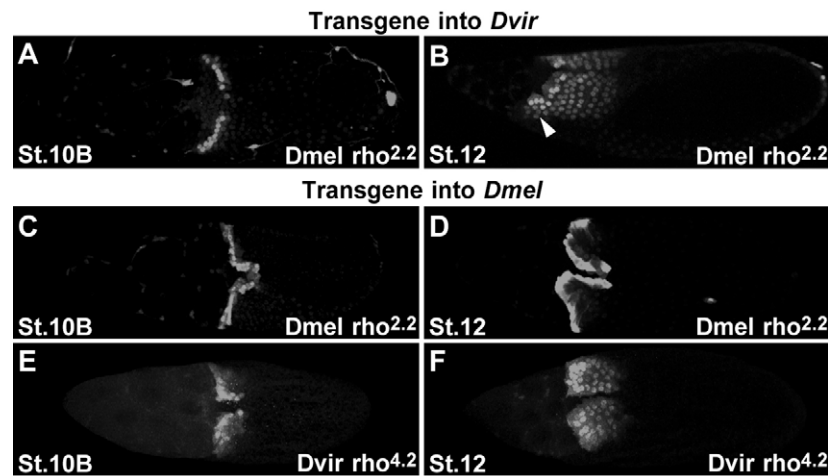
**Fig. 2. *Dvir rho*<sup>4.2</sup> carries a minimal enhancer activity accounting for the endogenous *rho* expression pattern during DA formation.** (A,B) *D. virilis rho* mRNA was detected in a V-shaped stripe missing its apex at stage 10B (A), and at stage 12 its distribution was restricted to the four domains where the four DAs later form (B). (C,D) The *Dvir rho*<sup>4.2</sup> enhancer drove the expression of GFP in a pattern similar to that of the endogenous *rho* in *D. virilis*. At stage 10B, a V-shaped stripe missing its apex was observed (C), and then at stage 12, the GFP reporter was expressed where the four DAs would later form (D). White arrowheads and brackets indicate the cells forming the anterior and posterior DAs, respectively (B,D). (E–G) Confocal images of the GFP reporter expression driven by the *Dvir rho*<sup>4.2</sup> enhancer and of endogenous Broad-Complex (BR-C) expression. The expression of *Dvir rho*<sup>4.2</sup>-GFP (green in E and G) and BR-C (magenta in F and G) was mutually exclusive at stage 10B. G is a merged image of E and F.

### Changes in the trans-regulatory landscape could cause the evolutionary divergence in *rho* expression

Phylogenetic analyses indicated that four-DA eggs are ancestral compared with two-DA eggs (Fig. 1A) (Powell, 1997; Throckmorton, 1962). This suggests that changes occurred in the trans-acting landscape regulating the cis-regulatory elements of *rho* and/or in these cis-regulatory elements themselves during the evolution from the four- to two-DA eggs. We assumed that if divergence of the trans-regulatory landscape was wholly responsible for the species-specific expression patterns of *rho*, the exogenous *rho* enhancers would adopt the same expression pattern as the endogenous *rho* expression (i.e. in *D. virilis*, *Dmel rho*<sup>2.2</sup> should be activated in a similar pattern to *Dvir rho*<sup>4.2</sup>). By contrast, if evolutionary modifications of the cis-regulatory elements were responsible for the divergence in *rho* expression, the activation pattern of *Dvir rho*<sup>4.2</sup> and *Dmel rho*<sup>2.2</sup> in the heterologous species should match the expression pattern of the endogenous *rho* in their species of origin (i.e. their homologous species).

To address this issue, we introduced the *Dmel rho*<sup>2.2</sup>-GFP reporter construct into *D. virilis*. As reported previously, *Dmel rho*<sup>2.2</sup> was activated in L-shaped domains at stages 10B and 12 in *D. melanogaster* (Dorman et al., 2004). Interestingly, *Dmel rho*<sup>2.2</sup> was activated in the V-shape with its apex missing in *D. virilis* at stage 10B (Fig. 3A). This pattern was very similar to the expression patterns of *Dvir rho*<sup>4.2</sup>-GFP and the endogenous *rho* in *D. virilis* at this stage. Furthermore, at stage 12, *Dmel rho*<sup>2.2</sup> drove expression in a pattern resembling that of *Dvir rho*<sup>4.2</sup>-GFP and endogenous *rho* in *D. virilis*, which was significantly different from the activation pattern of the *Dmel rho*<sup>2.2</sup> in its homologous species, *D.*





**Fig. 3. The trans-regulatory landscape controlling *rho* expression diverged between *D. virilis* and *D. melanogaster*.** (A,B) In *D. virilis*, the *Dmel rho*<sup>2.2</sup> enhancer drove expression of the reporter in a pattern resembling that of the endogenous *Dvir rho*<sup>4.2</sup> enhancer during DA formation; the V-shaped stripe at stage 10B (A) and the pattern corresponding to the four clusters of DA-forming cells at stage 12 (B) were observed. The cluster of cells forming the anterior DA is indicated by a white arrowhead (B). (C-F) *Dmel rho*<sup>2.2</sup> and *Dvir rho*<sup>4.2</sup> expressed in *D. melanogaster* were activated in a largely similar pattern, with some exceptions. The expression of *Dmel rho*<sup>2.2</sup>-GFP was detected as the L-shaped stripe on either side of the midline from stage 10B (C) to 12 (D) in *D. melanogaster*. When expressed in *D. melanogaster*, *Dvir rho*<sup>4.2</sup>-GFP showed the L-shaped expression pattern, similar to *Dmel rho*<sup>2.2</sup> in this species, but with a slightly posterior expansion (E). This extra expression of *Dvir rho*<sup>4.2</sup>-GFP further expanded posteriorly until stage 12 (F). Anterior is to the left; (A,C-F) dorsal view; (B) dorso-lateral view.

*melanogaster* (Fig. 3B). These results suggested that the *Dmel rho*<sup>2.2</sup> enhancer could respond to the heterologous trans-regulatory landscape in a manner similar to the endogenous *rho* enhancer in *D. virilis* during DA formation. Given these results, we speculated that diversification in the trans-regulatory landscape, rather than modification of the cis-acting elements, was mostly responsible for the differences in *rho* expression patterns during DA formation in these two species.

We then introduced *Dvir rho*<sup>4.2</sup>-GFP into *D. melanogaster* to examine whether our hypothesis held true in the reciprocal situation. In *D. melanogaster*, both *Dmel rho*<sup>2.2</sup> (Fig. 3C) and *Dvir rho*<sup>4.2</sup> (Fig. 3E) drove GFP expression in the L-shaped pattern at stage 10B. This result was consistent with our idea that the trans-regulatory landscape diverged significantly between *D. virilis* and *D. melanogaster*, and this divergence was responsible for the evolutionary changes in the *rho* expression patterns in these species.

### Evolution of the cis-regulatory elements also contributed to the species-specific activation patterns of the *rho* enhancers

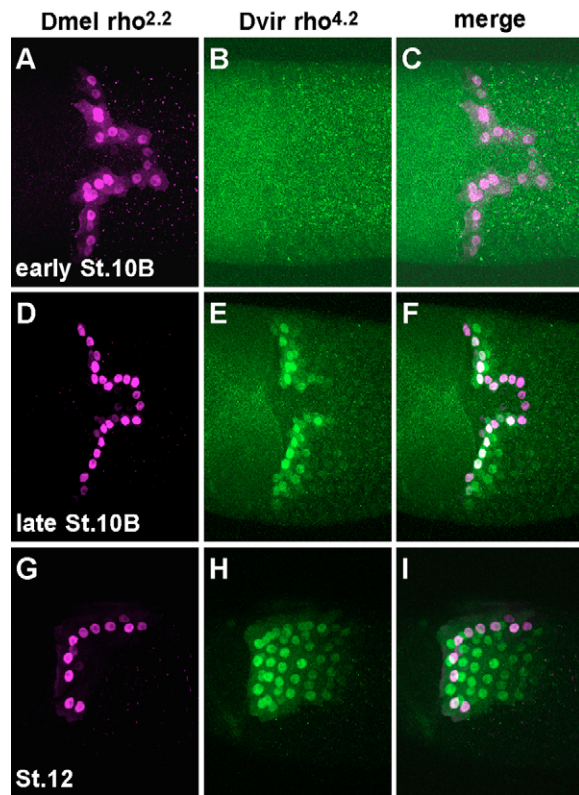
Our results suggested that the trans-regulatory landscapes that direct *rho* expression are evolutionarily divergent between *D. virilis* and *D. melanogaster*. However, we also noted that the expression domain of the *Dvir rho*<sup>4.2</sup>-GFP was slightly expanded posteriorly compared with that of the *Dmel rho*<sup>2.2</sup>-GFP in *D. melanogaster* (compare Fig. 3E with Fig. 3C). This difference became more obvious at stage 12 (compare Fig. 3F with Fig. 3D). At this stage, the *Dvir rho*<sup>4.2</sup>-GFP was expressed in a square on either side of the midline, rather than in the L-shaped pattern (Fig. 3F).

To study further the differential activation patterns between *Dvir rho*<sup>4.2</sup> and *Dmel rho*<sup>2.2</sup> in *D. melanogaster*, we introduced *Dvir rho*<sup>4.2</sup>-GFP and *Dmel rho*<sup>2.2</sup>- $\beta$ -Gal simultaneously into *D. melanogaster*, and observed the expression patterns driven by *Dvir rho*<sup>4.2</sup> and *Dmel rho*<sup>2.2</sup> with anti-GFP and anti- $\beta$ -Gal antibody staining, respectively. Both the GFP and  $\beta$ -Gal products of the reporter constructs carry a nuclear localization signal. Thus, the

expression of these reporters was detected as nuclear staining at the single-cell level. The expression of *Dvir rho*<sup>4.2</sup>-GFP and *Dmel rho*<sup>2.2</sup>- $\beta$ -Gal differed temporally and spatially (Fig. 4). When *Dmel rho*<sup>2.2</sup>- $\beta$ -Gal expression started, early in stage 10B, the *Dvir rho*<sup>4.2</sup>-GFP expression was not yet detectable (Fig. 4A-C). This difference was not owing to features of the GFP and  $\beta$ -Gal reporters (for example, their relative translational efficiencies), because when the GFP and  $\beta$ -Gal reporters were expressed under the control of the same enhancer, *Dmel rho*<sup>2.2</sup>, their expression patterns were essentially identical, temporally and spatially (see Fig. S1 in the supplementary material). Late in stage 10B, both *Dvir rho*<sup>4.2</sup>-GFP and *Dmel rho*<sup>2.2</sup>- $\beta$ -Gal were detectable (Fig. 4D-F). The anterior border of the expression domain of *Dvir rho*<sup>4.2</sup>-GFP and *Dmel rho*<sup>2.2</sup>- $\beta$ -Gal was the same (Fig. 4F). However, whereas *Dmel rho*<sup>2.2</sup>- $\beta$ -Gal was expressed in a single row of cells (Fig. 4D), *Dvir rho*<sup>4.2</sup>-GFP was expressed in one or two extra rows posterior to the single row where both reporters were expressed (Fig. 4E). At stage 12, expression of the *Dmel rho*<sup>2.2</sup>- $\beta$ -Gal was still restricted to the single row of cells (Fig. 4G), whereas the expression domain of the *Dvir rho*<sup>4.2</sup>-GFP had expanded posteriorly (Fig. 4H). These results suggest that the functions of the *rho* enhancers also evolved between these two species, although both enhancers maintained the ability to respond to the positional information that defines the anterior borders of their activation. However, we also noted that a few of the anterior-most cells expressed *Dvir rho*<sup>4.2</sup>-GFP but not *Dmel rho*<sup>2.2</sup>- $\beta$ -Gal at stage 12 (Fig. 4I), suggesting that some other functions might have also changed between the two enhancers.

### Conservation of the Grk signal during evolution of the DA number

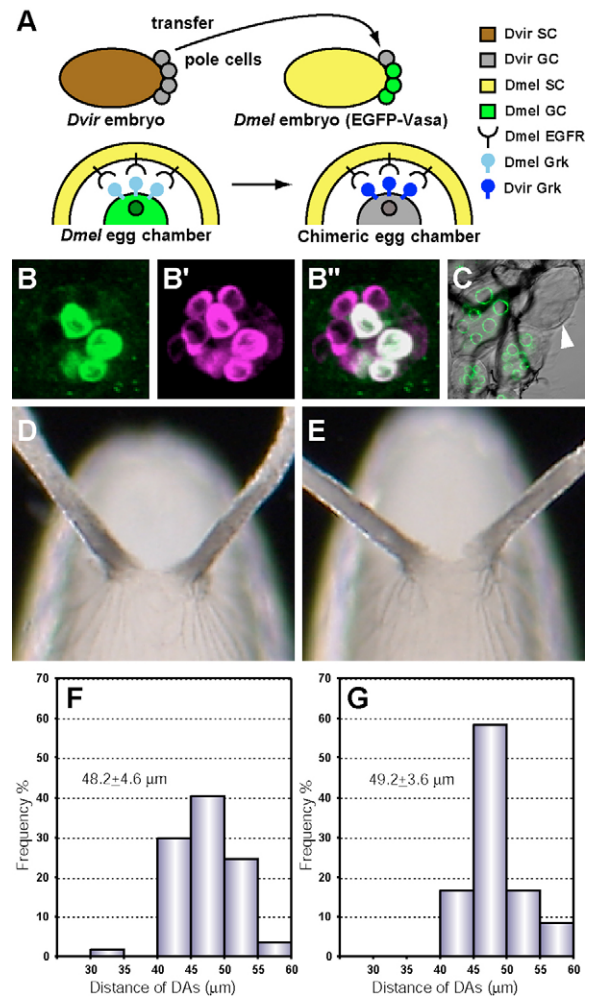
During DA patterning, the germline oocyte provides positional information to the somatic follicle cells through the Grk signal (Nilson and Schupbach, 1999). A mathematical model predicts that the distribution and amounts of Grk could play a key role in determining the number of DAs (Shvartsman et al., 2002). Considering that the Grk signal serves as the first cue for the



**Fig. 4. Function of the *rho* enhancer also diverged between *D. melanogaster* and *D. virilis*.** (A-I) *D. melanogaster* transgenic flies carrying both Dmel  $\rho^{2.2}$ - $\beta$ -Gal (magenta in A,C,D,F,G,I) and Dvir  $\rho^{4.2}$ -GFP (green in B,C,E,F,H,I) reporters were generated. The activities of the two enhancers were then determined at different developmental stages: early in stage 10B (A-C), late in stage 10B (D-F) and stage 12 (G-I). (A-C) At early stage 10B, Dmel  $\rho^{2.2}$ - $\beta$ -Gal (A) was activated, but Dvir  $\rho^{4.2}$ -GFP (B) was not yet active (C). (D-F) Late in stage 10B, Dvir  $\rho^{4.2}$ -GFP expression began (E) in a pattern similar to that of Dmel  $\rho^{2.2}$ - $\beta$ -Gal (D). The domain of Dvir  $\rho^{4.2}$ -GFP activation (E) largely overlapped with that of Dmel  $\rho^{2.2}$ - $\beta$ -Gal (D), although a slight posterior expansion of Dvir  $\rho^{4.2}$ -GFP was seen (F). (G-I) The anterior boundaries of the Dmel  $\rho^{2.2}$ - $\beta$ -Gal (G) and Dvir  $\rho^{4.2}$ -GFP (H) activation domains were similar, but the posterior expansion of Dvir  $\rho^{4.2}$ -GFP expression became prominent (I). The right panels are merged images of the left and middle panels.

positional information that defines the expression patterns of *rho* in the follicle cells, we proposed the following two hypotheses: first, that some divergence in the pivotal mechanism for determining the number of DAs occurred in the Grk signal itself, and second, that the trans-regulatory landscape that modifies the Grk signal in somatic follicle cells changed.

To distinguish between these possibilities, we generated a chimeric egg chamber that consisted of the *D. virilis* oocyte and *D. melanogaster* follicle cells (Fig. 5A). If the Grk signal diverged among *Drosophila* species, such a chimeric egg chamber would result in *D. virilis*-like appendages. The germline progenitors, or pole cells, were transplanted from *D. virilis* donor embryos into *D. melanogaster* host embryos carrying the EGFP-*vasa* construct, which expresses GFP in the germline (Sano et al., 2002). In the *D. melanogaster* hosts, the *D. virilis* germline was recognized by the absence of GFP expression.



**Fig. 5. Conservation of the Grk signal in *D. melanogaster* and *D. virilis*.** (A) The experimental scheme for interspecific pole cell transplantation from *D. virilis* to *D. melanogaster* embryos. (Top) Pole cells were transplanted from *D. virilis* donor embryos into *D. melanogaster* host embryos carrying the EGFP-*vasa* construct. (Bottom) Left: a normal egg chamber composed of an EGFP-*Vasa*-positive *D. melanogaster* oocyte and *D. melanogaster* somatic follicle cells. The *D. melanogaster* Grk activated *D. melanogaster* EGFR in this egg chamber. Right: a chimeric egg chamber. In the egg chamber of a *D. melanogaster* host female, the oocyte was replaced with one of *D. virilis* origin. In this chimeric egg chamber, the *D. virilis* Grk activated the *D. melanogaster* EGFR and directed the DA formation. (B-B'') A gonad of a *D. melanogaster* host embryo with transplanted *D. virilis* pole cells. The host embryos were stained with anti-GFP (B, green) and anti-*Vasa* (B', magenta) antibodies. The *D. melanogaster* host pole cells expressed both GFP and *Vasa* (B, green; B'', white), whereas the transplanted *D. virilis* pole cells expressed only *Vasa* (B', B'', magenta). The *D. virilis* pole cells were located within the gonad of the *D. melanogaster* host (B'', magenta). B'' is a merged image of B and B'. (C) Ovary of a *D. melanogaster* host female. GFP-positive host germline (green) and GFP-negative *D. virilis* germline (white arrowhead) cells are shown. The *D. virilis* germline was surrounded by *D. melanogaster* host follicle cells. (D,E) The chimeric egg (negative for EGFP, data not shown) had two DAs (E) identical to those of the wild-type egg (D). (F,G) The percentages of eggs in which the DA roots were separated by the distances indicated in the horizontal axes are shown. The two DAs were separated by a mean distance of  $48.2 \pm 4.6 \mu\text{m}$  ( $n=57$ ) in the wild-type (F) and  $49.2 \pm 3.6 \mu\text{m}$  ( $n=12$ ) in the chimeric (G) eggshells. The differences were not significant ( $0.5 > P > 0.2$ , Student's *t*-test).

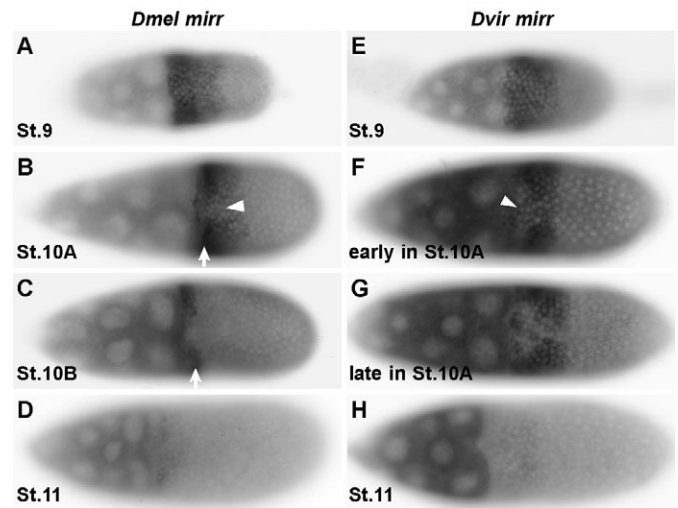
First, we examined whether the *D. virilis* pole cells could enter the gonad of the *D. melanogaster* embryo and whether the *D. virilis* germline cells could form the chimeric egg chamber in concert with *D. melanogaster* follicle cells in *D. melanogaster* ovaries (Fig. 5B–B",C). The transplanted *D. virilis* pole cells, which expressed only endogenous Vasa (Fig. 5B", purple), were incorporated into the gonad of *D. melanogaster* together with the *D. melanogaster* pole cells (Fig. 5B"). Furthermore, these germline cells derived from *D. virilis* formed a chimeric egg chamber together with the *D. melanogaster* follicle cells (white arrowhead in Fig. 5C), despite their phylogenetic divergence. In contrast to the prediction from the mathematical model, we found that all the chimeric eggs ( $n=16$ ) observed in four host females had two DAs (Fig. 5E), identical to the pattern of *D. melanogaster* (Fig. 5D).

This result suggests that the germline Grk signal did not change significantly during the evolution from four- to two-DA eggs. However, this interspecific transplantation could have introduced a non-specific effect that allowed the chimeric egg to develop two DAs, even if the Grk signal had diversified between the two species. For example, an incompatibility between the *D. virilis* Grk and the *D. melanogaster* EGFR could compensate for a difference in Grk signals. To address this problem, we took advantage of the fact that the Grk signal has two roles during oogenesis (Nilson and Schupbach, 1999). In addition to its role in DA formation, the Grk signal has an essential role in the dorsal-ventral patterning of the egg (Neuman-Silberberg and Schupbach, 1993; Nilson and Schupbach, 1999). When the dorsal-ventral patterning is aberrant in *D. melanogaster*, the DAs are shifted with respect to the dorsal midline, dorsally or ventrally, respectively, with the loss or gain of Grk signaling (Neuman-Silberberg and Schupbach, 1993; Nilson and Schupbach, 1999; Schupbach, 1987). Therefore, we measured the distance between the roots of the two DAs in wild-type and chimeric eggshells, both of which were laid by a single transplanted host female to avoid any other effects, such as nutritional status. The distances between the two DAs in the wild-type ( $48.2 \pm 4.6 \mu\text{m}$ ,  $n=57$ ) and chimeric ( $49.2 \pm 3.6 \mu\text{m}$ ,  $n=12$ ) eggshells showed no statistically significant difference (Fig. 5F,G,  $0.5 > P > 0.2$ , Student's *t*-test), suggesting that the dorsal-ventral patterning occurred normally in the chimeric eggs. It is unlikely that the two different patterning events, DA formation and dorsal-ventral patterning, both of which are triggered by Grk signal-dependent positional information, were both properly compensated for by chance. Thus, we speculate that the role of Grk in DA patterning is conserved between *D. virilis* and *D. melanogaster*, suggesting that subsequent events, downstream of the Grk signal, diverged to elicit the different expression of *rho* between these two species.

### Mirr is a candidate molecule responsible for the divergence of *rho* expression

In *D. melanogaster*, *mirr*, which encodes a homeobox transcription factor, is expressed in dorsal anterior follicle cells (Jordan et al., 2000; Zhao et al., 2000). This expression pattern depends on the Grk signal, and *mirr* subsequently activates the expression of *rho* during oogenesis (Jordan et al., 2000; Jordan et al., 2005; Zhao et al., 2000). Therefore, *mirr* might introduce the evolutionary changes into the landscape of trans-regulatory factors that are responsible for the divergence of *rho* expression in *D. virilis* and *D. melanogaster*. To test this hypothesis, we analyzed the *mirr* expression patterns in these two species.

In both species, *mirr* expression began similarly, at the dorsal-anterior region of the follicle cells, at stage 9, prior to the onset of *rho* expression (Fig. 6A,E). After this stage, the *mirr*



**Fig. 6. The expression pattern of *mirr* diverged evolutionarily between *D. melanogaster* and *D. virilis*.** (A–H) The expression of *mirr* was detected by in situ hybridization during the DA formation in *D. melanogaster* (A–D) and *D. virilis* (E–H). (A) *D. melanogaster mirr* was expressed as a single broad domain at the dorsal-anterior region of the follicle cells at stage 9. (B) At stage 10A the *D. melanogaster mirr* expression was repressed in the dorsal midline (white arrowhead) and elevated in the cells at the anterior border of this expression domain (white arrow). By stage 10B, the *mirr* expression was restricted to the single row of anterior cells on either side of the midline (white arrow in C), and then was barely detected at stage 11 (D). (E) *D. virilis mirr* was expressed at stage 9 at the dorsal-anterior region of the follicle cells, like *D. melanogaster mirr*. (F) The *mirr* expression was gradually lost from the anterior region of its expression domain (white arrowhead) early in stage 10A. (G) By late in stage 10A, the *D. virilis mirr* expression was resolved into two dorso-lateral domains, similar to *D. virilis rho* expression at the same stage (see Fig. 1E). Note that the *mirr* expression was absent from a triangle at the anterior midline region of the follicle cells. (H) The expression was almost absent at stage 11.

expression followed species-specific patterns, which resembled the *rho* expression pattern in each species. In *D. melanogaster*, the expression of *mirr* was repressed in the dorsal midline at stage 10A (white arrowhead in Fig. 6B) and was elevated in the follicle cells at the anterior boundary (white arrow in Fig. 6B), which corresponded to the anterior row of *rho*-expressing cells in the L-shaped pattern at stage 10B (Fig. 1D). This anterior expression continued until stage 10B (white arrow in Fig. 6C). By contrast, early in stage 10A in *D. virilis*, the *mirr* expression was repressed only at the dorsal-anterior region, not the entire dorsal midline (white arrowhead in Fig. 6F). Late in stage 10A, the region lacking *mirr* expression expanded and became a triangle (Fig. 6G), which consequently divided the *mirr*-expressing region into two dorsal-lateral domains that were reminiscent of the *rho*-expressing domains of *D. virilis* at stage 10A (Fig. 1E). After stage 10B, the expression of *mirr* was barely detectable in either species (Fig. 6D,H). The expression of *D. virilis mirr* was not restricted to the domains expressing *rho* (Fig. 1E). Therefore, the expression of *mirr* does not always cause the expression of *rho*. Nevertheless, our results suggest evolutionary changes in the *mirr* expression patterns as a candidate mechanism for the divergence of *rho* expression at stage 10A in *D. virilis* and *D. melanogaster*.



## DISCUSSION

### Role of the trans-regulatory landscape in the evolutionary diversification of DA numbers

Changes in gene expression patterns during evolution can be attributed to two distinct mechanisms. First, alterations in the cis-regulatory sequence of a gene can be responsible for the divergence of its expression pattern. Second, changes in trans-regulatory factors can cause gene expression patterns to diverge, even if the cis-regulatory elements of these genes are conserved during evolution. Diversification in enhancer elements is known to contribute predominantly to the evolution of animal morphology (Belting et al., 1998; Gompel et al., 2005; Wittkopp et al., 2002). The gain and loss of cis-acting elements have played central roles in the divergence of the expression patterns of genes that play crucial roles in the generation of specific characteristics in different species (Gompel et al., 2005). In this study, we investigated the contribution of these two processes to the evolutionary diversification of DA numbers in *D. virilis* and *D. melanogaster*. In addition to the importance of cis-regulatory elements demonstrated previously, our findings suggest that the landscape of trans-regulatory factors could also change and affect morphological divergence during evolution.

In *D. melanogaster*, *rho* expression has an instructive role in defining the pattern of DA precursor cell formation (Sapir et al., 1998; Ward and Berg, 2005). In addition, we previously demonstrated that the expression patterns of *rho* diverged and were correlated with the position and number of DAs in *D. virilis* and *D. melanogaster* (Nakamura and Matsuno, 2003). Therefore, in this study, we mostly focused on the enhancers of *rho* in these species. To distinguish whether divergence in the trans-regulatory landscape or the cis-regulatory elements is important for the evolutionary change in *rho* expression patterns between *D. melanogaster* and *D. virilis*, we introduced reporter constructs of Dvir rho<sup>4.2</sup> and Dmel rho<sup>2.2</sup> into these two species. Phylogenetic analyses of *Drosophila* species suggest that the four DAs are an ancestral characteristic, and that the flies with two DAs evolved from four-DA ancestors (Fig. 1). Thus, the characteristics of Dmel rho<sup>2.2</sup> were probably derived from the ancestral Dvir rho<sup>4.2</sup> enhancer. We found that Dvir rho<sup>4.2</sup> and Dmel rho<sup>2.2</sup> adopted the expression pattern of the endogenous *rho* of the heterologous species. These results suggest that Dvir rho<sup>4.2</sup> and Dmel rho<sup>2.2</sup> did not diverge in terms of their ability to respond to the trans-acting factors in follicle cells. Therefore, we speculated that changes in the cis-regulatory elements from Dvir rho<sup>4.2</sup> to Dmel rho<sup>2.2</sup> were not the main cause for divergence in the activation patterns of these enhancers in their homologous species.

Although the DNA sequences of Dvir rho<sup>4.2</sup> and Dmel rho<sup>2.2</sup> diverged drastically, several putative binding sites for transcription factors, such as ETS, Su(H) and BR-C, were common to both (data not shown), which could explain the conserved function of the two enhancers. Recently, it was reported that BR-C represses the activity of Dmel rho<sup>2.2</sup> in a cell-autonomous manner during DA patterning (Ward et al., 2006), and this repression allows the enhancer to be activated in the L-shaped region. These two *rho* enhancers share five overlapping binding sites for BR-C (data not shown). Thus, these BR-C-binding sites might serve as cis-regulatory elements to transmit the conserved functions of these two enhancers. Notch (N) signaling also regulates Dmel rho<sup>2.2</sup> (Ward et al., 2006), and we found that one binding site for Su(H) is conserved among all six *Drosophila* species examined (data not shown). Conservation of the binding sites for these various transcription factors and their possible involvement in the evolution of DA patterning suggest that *rho* expression is controlled by complex responses to multiple

transcription factors, instead of by a simple EGFR-signal feedback system, which is consistent with the model proposed by Peri and Roth (Peri and Roth, 2000).

We identified *Mirr* as a candidate for the difference in the landscape of trans-regulatory factors between *D. melanogaster* and *D. virilis*. The distribution of the *mirr* transcript was significantly different between these species. *mirr* induces *rho* expression, and regulates N signaling by repressing *fringe*, probably thereby regulating *rho* (Blair, 2000; Bruckner et al., 2000; Moloney et al., 2000). Although whether or not *Mirr* function is also involved in the regulation of *rho* transcription in *D. virilis* remains to be tested, it is conceivable that changes in the expression patterns of *mirr* may account, at least in part, for the divergence in the activation patterns of Dvir rho<sup>4.2</sup> and Dmel rho<sup>2.2</sup> in *D. melanogaster* and *D. virilis*.

In *D. melanogaster*, *rho* is expressed in a saddle-shaped pattern at stage 10A. We analyzed the genomic region within 26.2-kb upstream and 11.8-kb downstream of the transcription initiation site of *rho*, but failed to identify an enhancer element responsible for this early expression pattern (data not shown). The function of this early *rho* expression in DA formation has not yet been studied. Therefore, we could not exclude the possibility that an enhancer that regulates the early expression of *rho* is involved in the diversification of the *rho* expression pattern. However, we speculate that this early expression of *rho* does not play a significant role in determining the number of DAs, because *D. pseudoobscura* and *D. melanica* have eggs with two DAs, but the saddle-shaped pattern of *rho* expression was not detected in these species (T.K., Y.N. and K.M., unpublished). Therefore, the subsequent expression of *rho* is probably what plays a crucial role in determining the DA number.

### Changes in cis-acting elements may contribute to the evolutionary divergence in *rho* expression between *D. melanogaster* and *D. virilis*

Our present analysis revealed that the functions of Dvir rho<sup>4.2</sup> and Dmel rho<sup>2.2</sup> are largely conserved. However, we also found that Dmel rho<sup>2.2</sup> had evolved a novel trait during its diversification from Dvir rho<sup>4.2</sup>. In *D. melanogaster*, both Dvir rho<sup>4.2</sup> and Dmel rho<sup>2.2</sup> were activated in the L-shaped pattern at stage 10B. However, Dvir rho<sup>4.2</sup> was activated in one or two extra rows of cells posterior to the single row of cells where Dmel rho<sup>2.2</sup> was active at this stage. At stage 12, Dvir rho<sup>4.2</sup> was activated much more posteriorly, although Dmel rho<sup>2.2</sup> was still active only in the single row of cells. Given that Dvir rho<sup>4.2</sup> is ancestral to Dmel rho<sup>2.2</sup>, we speculate that Dmel rho<sup>2.2</sup> lost a cis-acting element capable of being activated in this posterior region, or gained a cis-acting element that suppresses its activity in this region at stage 12. Indeed, it is likely that this posterior activation of *rho* is an ancestral characteristic, because the endogenous expression of *rho* in this region is found in *D. virilis* but not *D. melanogaster*.

### The Grk signal may not play a crucial role in the evolution of DA number in *D. melanogaster* and *D. virilis*

For the formation of DAs, the patterning of EGFR signaling activity in the follicle cells plays crucial roles in *D. melanogaster* (Wasserman and Freeman, 1998). Two major events are involved in the regulation of EGFR signaling activity in these cells. First, Grk specifically localizes to the dorsal anterior part of the oocyte and activates EGFR in the overlying follicle cells (Wasserman and Freeman, 1998). Second, in the follicle cells, positive and negative feedback loops elaborate the pattern of EGFR signaling activity that

ultimately determines the number of DAs (Wasserman and Freeman, 1998). Thus, the first and second events are germ- and soma-derived events, respectively.

As predicted from the above model, the intensity of Grk expression and the width of its expression domain in the oocyte are thought to define the number of DAs (Shvartsman et al., 2002). A mathematical study predicted that changes in the amount and distribution of Grk protein in the oocyte can account for the evolution of eggshells with zero to four DAs in *Drosophila* species (Shvartsman et al., 2002). However, our experiments involving a chimeric egg chamber suggest that changes in the follicle cells, but not in the oocyte, have an instructive role in determining the number of DAs. These results suggest that the change in Grk signaling did not contribute to the evolution of DA numbers in these species. This is consistent with a previous finding that the distribution and amount of *grk* mRNA do not show a significant difference between *D. melanogaster* and *D. virilis* (Peri et al., 1999). However, our results do not exclude the possibility that changes in Grk signaling play major roles in the diversification of DA numbers during the evolution of other *Drosophila* species.

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#### Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/134/8/1529/DC1>

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