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The *Drosophila* JNK Pathway Controls the Morphogenesis of the Egg Dorsal Appendages and Micropyle

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During *Drosophila* oogenesis, the formation of the egg respiratory appendages and the micropyle require the shaping of anterior and dorsal follicle cells. Prior to their morphogenesis, cells of the presumptive appendages are determined by integrating dorsal–ventral and anterior–posterior positional information provided by the epidermal growth factor receptor (EGFR) and Decapentaplegic (Dpp) pathways, respectively. We show here that another signaling pathway, the *Drosophila* Jun-N-terminal kinase (JNK) cascade, is essential for the correct morphogenesis of the dorsal appendages and the micropyle during oogenesis. Mutant follicle cell clones of members of the JNK pathway, including *DJNKK/hemipterous* (*hep*), *DJNK/basket* (*bsk*), and *Djun*, block dorsal appendage formation and affect the micropyle shape and size, suggesting a late requirement for the JNK pathway in anterior chorion morphogenesis. In support of this view, *hep* does not affect early follicle cell patterning as indicated by the normal expression of *kekkon* (*kek*) and *Broad-Complex* (*BR-C*), two of the targets of the EGFR pathway in dorsal follicle cells. Furthermore, the expression of the TGF-β homolog *dpp*, which is under the control of *hep* in embryos, is not coupled to JNK activity during oogenesis. We show that *hep* controls the expression of *puckered* (*puc*) in the follicular epithelium in a cell-autonomous manner. Since *puc* overexpression in the egg follicular epithelium mimics JNK appendages and micropyle phenotypes, it indicates a negative role of *puc* in their morphogenesis. The role of the JNK pathway in the morphogenesis of follicle cells and other epithelia during development is discussed. **© 2001 Academic Press**

Key Words: **JNK; *hep*; morphogenesis; epithelium; oogenesis; follicle cells; appendages; micropyle.**

INTRODUCTION

The migration of cells, either isolated or in groups, is a fundamental process required during the morphogenesis of most body structures. The genetic control of these cellular behaviors and the basis of their diversity are poorly understood. Work in several organisms has revealed that cell–cell communication is crucial for the initiation and coordination of migratory processes. In particular, in *Drosophila*, the JNK MAPK pathway has been shown to be essential for the movement of different epithelia during development (Agnès *et al.,* 1999; Ip and Davis, 1998; Noselli, 1998; Noselli and Agnes, 1999). In embryos, activation of this signaling pathway is required for the convergent movement of two lateral epithelia toward the dorsal midline, leading to dorsal closure. Recent work has shown that, later in development, the *hemipterous* (*hep*)/*DJNKK* and *Dfos* genes are also required for the morphogenesis of epithelial sac-like structures, the imaginal discs (Agnès *et al.,* 1999; Zeitlinger and Bohmann, 1999). In this study, we show that members of the JNK pathway, including *hep*, *bsk*, *Djun*, and *puc*, play an essential role in the correct morphogenesis of the tubular structures that are derived from the egg follicular epithelium, the dorsal appendages (DA). These genes are also shown to be involved in the correct shaping and sizing of the egg sperm entry point, the micropyle.

During *Drosophila* oogenesis, extensive cell migration

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occurs to shape and organize the egg chambers (Spradling, 1993). Each egg unit is made of 16 germline cells organized in a syncitium or cyst, surrounded by a monolayered follicular epithelium (Dobens and Raftery, 2000). Within this relatively simple structure, the growing oocyte undergoes sequential polarization along the antero-posterior (AP) and dorso-ventral (DV) axes. Both of these key patterning events are initiated by the Gurken (Grk)/TGF-α ligand present in the oocyte which activates the epidermal growth factor receptor (EGFR) tyrosine kinase signaling pathway in the overlaying follicle cells (Gonzalez-Reyes *et al.,* 1995; Roth *et al.,* 1995). On the dorsal–anterior side, where the DA will eventually form, EGFR activation is first broad and subsequently refined into "twin peaks" on both sides of the egg, representing the precursors of the DA (Wasserman and Freeman, 1998). The positioning of the DA along the AP axis is controlled by a different pathway, the TGF-β/Dpp pathway (Peri and Roth, 2000; Twombly *et al.,* 1996). Thus, DA formation represents an attractive system for studying how different signaling pathways converge at the level of an epithelial sheet in order to build up differentiated structures.

Although much work has been done on the early events leading to the patterning of the dorsal follicular epithelium and the egg (Ray and Schupbach, 1996; van Eeden and Johnston, 1999), little is known about relaying genes and signaling pathways that shape the flat epithelium into an elaborate tubular structure. Here, we show that the JNK pathway plays an important role in DA and micropyle morphogenesis. Cells of the presumptive appendages that are mutant for *hep*, *bsk*, and *Djun* show unique phenotypes. Instead of elongating, cells stay clustered at their initial dorsal–anterior position, leading to strongly shortened appendages. We show that *hep* mutations do not affect the positioning nor the determination of precursors of the DA, suggesting that *hep* and the JNK pathway are specifically required for the morphogenesis of the dorsal epithelium at a late step during appendages development. We further show that *puc* is a target of *hep* in the follicle cells, and that overexpression of *puc* can mimic loss of *hep* function, consistent with its role as a MAPK phosphatase. Interestingly, we found that one of the targets of the JNK pathway during dorsal closure, the TGF-β homolog *dpp*, is not under the control of *hep* during follicle cell development, indicating tissue-specific variation in JNK signaling mechanism. DA and micropyle formation thus provide a novel model to study the link between patterning and JNK-dependent epithelial morphogenesis.

MATERIALS AND METHODS

Genetics

A description of genetic markers and chromosome balancers can be found in Lindsley and Zimm (1992). The generation and characterization of *hep* mutants has been described (Glise *et al.,* 1995). Homozygous follicle cell clones for *hep*, *bsk*, and *Djun* were induced by using the UAS-FLP method (Duffy *et al.,* 1998). *hep* mutant stocks (*y w hep^{r75} FRT101/FM7*) were crossed to *y w UBGFP FRT101;TM6B/e22cGAL4, UAS-FLP* (a gift from D. Bilder). The expression of the FLP recombinase in follicular cells using the *e22cGAL4* line induced cell type-specific mutant clones in the follicle cells, which were identified by the absence of GFP expression. For phenotypic rescue of mutant egg chambers, clones were induced in females carrying a *UBhep* (*y w hep^{r75} FRT101/year w UBGFP FRT101;UBhep/e22cGAL4, UAS-FLP*) or *UBlic* transgene (*y w hep FRT101/year w UBGFP FRT101;UBlic/e22cGAL4, UAS-FLP*) (Glise *et al.,* 1995; Suzanne *et al.,* 1999). *Basket* and *Djun* clones were induced by using the following mutant lines: *w;bsk^{170B} FRT40A/CyO* and *w;FRTG13 Djun^{2}/Cyo* (a gift from L. Kockel); the following stocks were used to generate clones in the follicle cells: *FRT40A/CyO;T155GAL4 UAS-FLP* and *FRTG13;T155GAL4 UAS-FLP*. The *E4*, *T155*, *CY2*, and *55B* GAL4 lines used in this study have been described elsewhere (Queenan *et al.,* 1997). The *slboGAL4* line is described in Rorth *et al.* (1998). The *puc^{E69}* line is described in Ring and Martinez Arias (1993). *puc^{B48}* was a gift from L. Dobens.

Immunohistochemistry and X-Gal Staining

Staining of egg chambers with X-gal or antibodies were performed as in Lasko and Ashburner (1990) and Suzanne *et al.* (1999). The following antibodies have been used: anti-BR-C (1:200, kindly provided by G. Guild), anti-Dfos (1:200, kindly provided by L. Kockel), anti-β-galactosidase (Cappel), anti-rabbit FITC (fluorescein)-tagged (1:200), anti-rat CY5 (1:200), and anti-mouse RedX (1:200) secondary antibodies (Jackson ImmunoResearch Laboratories). Confocal images were taken with either LSM10 Zeiss or Leica TCS NT confocal microscope. Other images were taken with Leica DC200 and Nikon Coolpix 990 digital cameras and processed by using Photoshop 5.0 (Adobe).

RESULTS

Hemipterous Is Required for DA and Micropyle Morphogenesis

The *hemipterous* (*hep*) gene is involved in the morphogenesis of different epithelia during embryonic and pupal development (Agnès *et al.,* 1999; Glise *et al.,* 1995). In adult females, the continuous formation of egg chambers represents an important source of newly assembled epithelia undergoing dramatic morphogenesis (Dobens and Raftery, 2000; Spradling, 1993). To determine whether the JNK pathway is involved in some aspect of follicle cell morphogenesis, we first examined the role of *hep* during oogenesis.

Females that are homozygous for the hypomorphic *hep^{1}* allele lay eggs that show an occasional reduction of the DA (not shown), suggesting that *hep* may have a role in the formation of these epithelial-derived structures. To analyze the function of *hep* in appendage formation more directly, we used the UAS-FLP system (Duffy *et al.,* 1998) to generate targeted mosaics in the follicle cells of strong loss-of-function *hep* mutations. Females bearing clones of the *hep^{r75}* allele (a null or strong hypomorphic *hep* mutation; Glise *et al.,* 1995) produce eggs with strongly reduced DA (Figs. 1B and 1C). The reduction of the size of the append-

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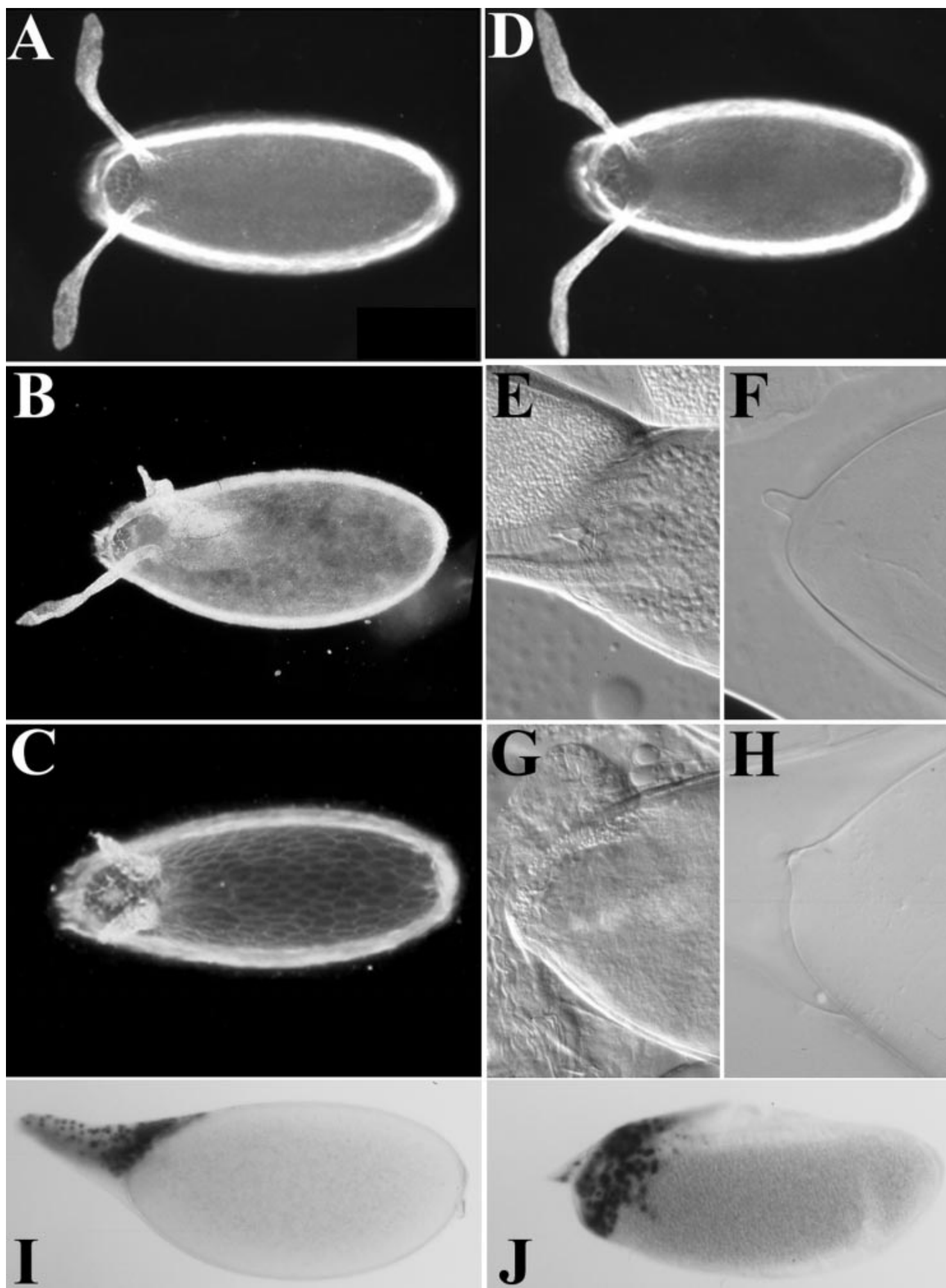

FIG. 1. *hep* controls DA and micropyle formation. Wild-type eggs have two DA that end in a paddle-like structure (A). In the absence of *hep* function in the anterior follicle cells, DA do not elongate, but instead cells accumulate at the bases of the appendages. Depending on the size and localization of the mutant clones, DA defects can be either partial (B) or complete and symmetrical (C). Expression of a UB-*hep* construct in the follicle cells completely rescues the DA phenotype; mother genotype: *y w hepr75 FRT101/y w UB-GFP FRT101;e22cGAL4, UAS-FLP/UB-hep* (D). Nomarski views of the anterior chorion (E, G) and vitelline membrane (F, H) of wild-type (E, F) and *hep* mutant (G, H) eggs. Some mutant eggs show a reduced micropyle (G, H). (G) Both the appendages and the micropyle are defective. (I, J) X-gal stainings of wild-type (I) and mutant (J) stage-14 egg chambers expressing the kekkon-lacZ marker to show the extent of appendage elongation. (I) Elongation is almost complete while in the mutant elongation is absent. (A–D) Dark field views. Dorsal is up and anterior is left. Mutant follicle cell clones were generated by using the following flies: *y w hep*^{r75} *FRT101/y w UB-GFP FRT101;e22cGAL4, UAS-FLP/+*, or *y w hep*^{r75} *FRT101/y w UB-GFP FRT101;e22cGAL4, UAS-FLP/kek-lacZ* (in J).

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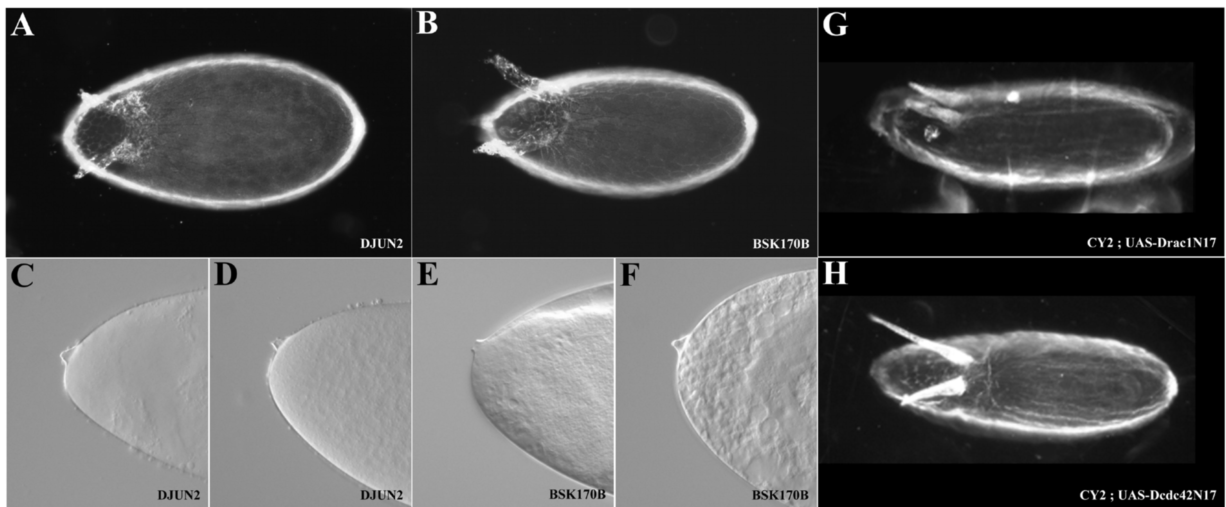

FIG. 2. *Basket*, *Djun*, *Drac1*, and *Dcdc42* control appendage and micropyle morphogenesis. Dark field (A, B) and Nomarski (C–F) views of eggs derived from *w;FRTG13, Djun^2/FRTG13;T155GAL4, UAS-FLP/+* (A, C, D) and *w;bsk^{170B}, FRT40A/FRT40A;T155GAL4, UAS-FLP/+* (B, E, F). The generation of *bsk* and *Djun* mutant follicle cell clones affects normal egg morphology, leading to shortened appendages (A, B) and shortening of the micropyle (C–F). These phenotypes are very similar to those derived from *hep* mutant clones (see Fig. 1). Dark field views of eggs derived from *CY2GAL4;*UAS-*Drac1N17* (G) or *CY2GAL4;*UAS-*Dcdc42N17* (H) females. Drac1N17 induces phenotypes that are similar to those obtained in other JNK pathway mutants, including the absence of elongation of appendages and a paddle-less phenotype. In the case of Dcdc42N17 overexpression (H), the phenotype is slightly different and results in the formation of thinner appendages showing irregular shape and bulging. This suggests that the two GTPases may play qualitatively different roles in appendage morphogenesis. Anterior is to the left.

ages, and the accompanying expansion of their basis, suggests an absence of appendages elongation resulting in a clustering of precursor cells during late stages of oogenesis. That this shortened appendage phenotype results from an aberrant elongation during late oogenesis (stages 12–14) is supported by the observation of abnormal egg chambers with shortened anterior ends (Figs. 1I and 1J). In the following, we will refer to elongation to describe the series of events transforming the appendages precursor cells, which are part of a flat epithelium in stage-10 egg chambers, into the fully elongated appendages that are observed in older stages and mature eggs. A similar phenotype (absence of full elongation) is also observed for the micropyle (the sperm entry point). In some of the eggs that are derived from *hep* mutant clones (hereafter referred to as *hep* mutant eggs or egg chambers), the micropyle is reduced in size and its shape adopts a more blunted aspect (Figs. 1G and 1H, and data not shown). The frequent observation of a simultaneous reduction of the appendages and the micropyle suggests that both structures derive from a common region, i.e., cells from the anterior main body follicles (see Fig. 1G and below).

Because *hep^{r75}* germline clones do not produce, or only very rarely (data not shown), DA defects, we conclude that *hep* is required in the follicular epithelium for DA and micropyle morphogenesis. These phenotypes can be fully rescued by expressing a *hep* cDNA using a ubiquitin-*hep* transgene (UB*hep*; Glise *et al.,* 1995; Fig. 1D). Rescue is not observed with another related stress-activated p38 MAPKK construct (UB-*lic*; Suzanne *et al.,* 1999; data not shown), indicating that *hep* is specifically required in the follicle cells for the morphogenesis of the DA and micropyle during oogenesis.

Members of the JNK Pathway Affect DA and Micropyle Morphogenesis

In order to test whether other components of the JNK pathway may also be involved in follicle cell morphogenesis, we performed UAS-FLP-mediated clonal analysis by using mutant alleles of the *basket* and *Djun* genes. *bsk* and *Djun* mutant clones led to a variety of DA phenotypes ranging from partial (data not shown) to complete absence of elongation (Figs. 2A and 2B). The phenotypes are very similar to *hep* mutant appendages, showing an expansion of the appendage bases to the expense of more distal parts. The analysis of dechorionated eggs showed micropyles with a reduced size, sometimes associated with a characteristic truncated shape (Figs. 2C–2F). In these examples, the micropyle looks incompletely "closed" (Figs. 2E and 2F), a phenotype reminiscent of the closure defects associated with JNK pathway mutants in embryos and adults (Agnès *et al.,* 1999). Interestingly, mutant follicle cell clones of the *licorne* gene, which encodes a related p38 MAPKK (Suzanne *et al.,* 1999), do not affect chorion morphogenesis (data not shown), showing that follicle cell development is specifi-

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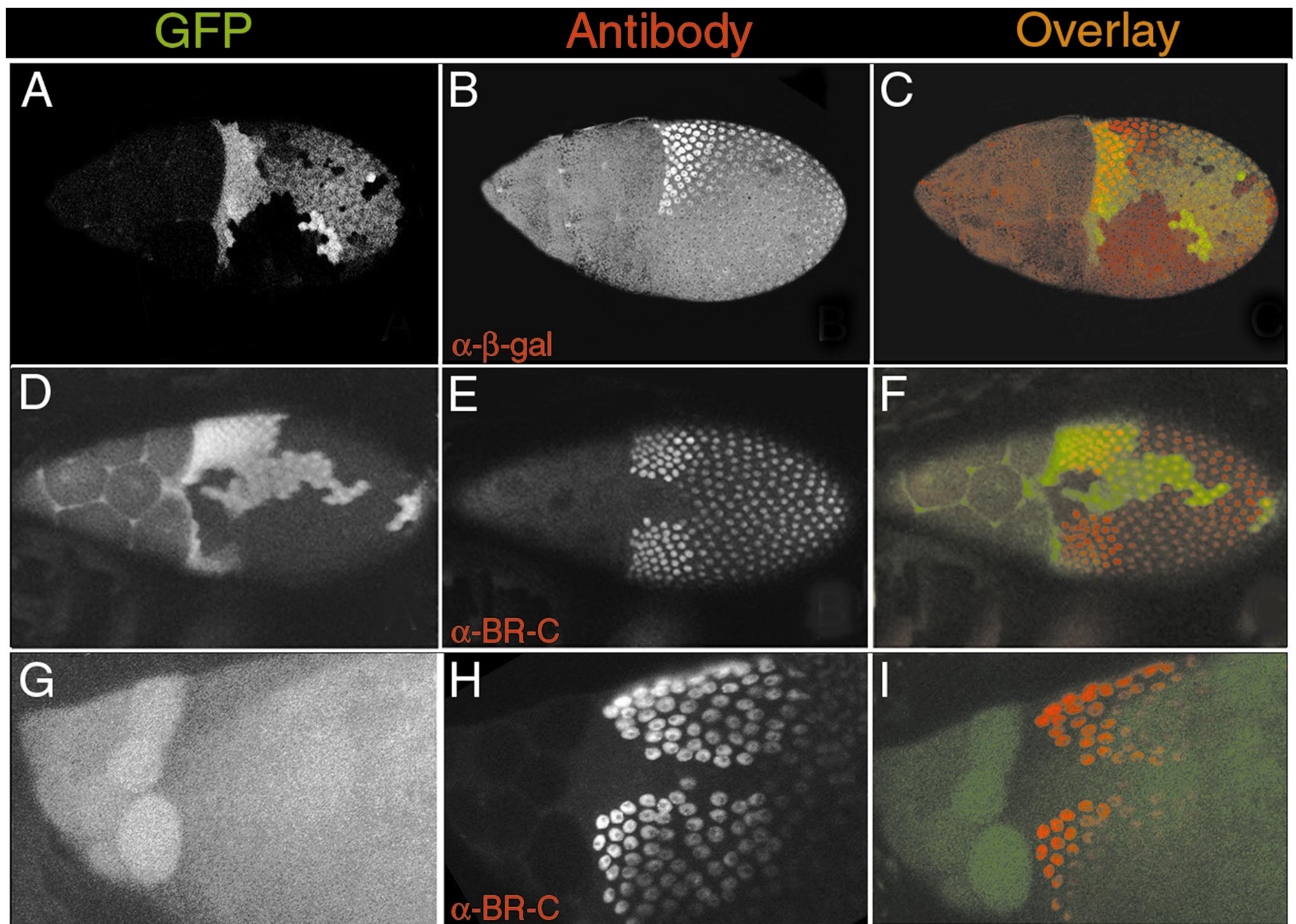

FIG. 3. The expression of BR-C and kekkon are normal in *hep* mutant clones. *hep* mutant follicle cell clones are generated by using the FRT/UAS-FLP method (see Materials and Methods; Duffy *et al.,* 1998) with the following flies: *y w hep*^{r75} *FRT101/UB-GFP FRT101; e22cGAL4, UAS-FLP/+*. Mutant clones are marked by the absence of GFP. (A–C) and (D–F) correspond to mosaic egg chambers, whereas the follicle cells in (G–I) are completely mutant. Ovaries were stained with either anti-β-galactosidase (to reveal *kek-lacZ* expression; B, C) or anti-BR-C (E, F, H, I) antibodies. The pattern of *kek* and BR-C expression is not affected in *hep* mutant clones, indicating that the patterning and positioning of appendage precursor cells are normal in JNK mutant egg chambers. (A–C) and (D–F) are stage-10 egg chambers, while (G–I) correspond to stages 12–13. (C), (F), and (I) are overlays of (A, B), (D, E), and (G, H), respectively. Anterior is to the left.

cally controlled by one (JNK) but not the other (p38) stress-activated MAPK pathway.

We also tested the role of more upstream components by expressing dominant negative forms of the small GTPases Drac1 (Drac1N17) and Dcdc42 (Dcdc42N17; referred to as DracDN and Dcdc42DN, respectively; Luo *et al.,* 1994). Expression of these molecules in the embryo can mimic a loss of *hep* or *basket* function (Harden *et al.,* 1995; Riesgo-Escovar *et al.,* 1996). Expression of DracDN in the follicular epithelium (using the *T155Gal4* driver, which is expressed in all the follicle cells) leads to the formation of short DA that resemble those generated by *hep*, *bsk*, and *Djun* clones (Fig. 2G). Although expression of Dcdc42DN in the follicle cells also perturbs DA formation, the defects are slightly different (Fig. 2H). Most of the DA have a normal size, but are thinner and irregular in shape. The different effects generated by Drac1DN and Dcdc42DN suggest that these molecules may perform specific functions during morphogenesis, as observed in other tissues like muscles and imaginal discs (Agnès *et al.,* 1999; Glise and Noselli, 1997; Luo *et al.,* 1994).

Together, these data indicate that the canonical JNK pathway is required during oogenesis for the normal morphogenesis of DA and the micropyle.

Hemipterous Does Not Affect the Patterning of Appendage Precursor Cell

The absence of appendage formation in *hep* and other JNK pathway mutant eggs may originate from either aber-

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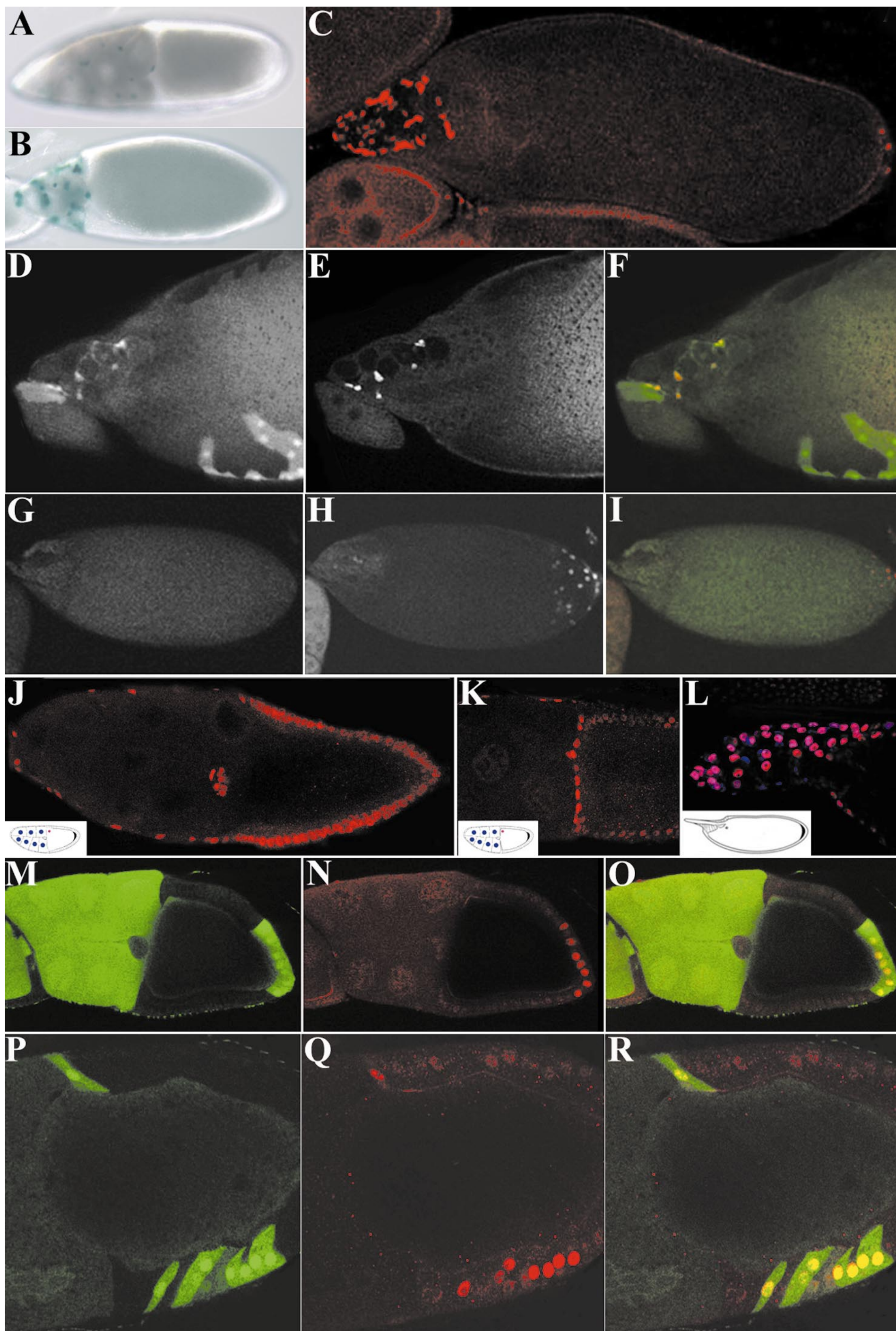

FIG. 4. *puc* is a target of *hep* in follicle cells. Expression of the puc^{E69} enhancer-trap at stages 10B (A), 12 (B), and 13–14 (C). Egg chambers were stained with either X-Gal (A, B) or anti-β-galactosidase antibodies to reveal *puc-lacZ* expression (C–R). In an egg chamber that is completely mutant for *hep* (G), puc^{E69} expression is completely abolished at the anterior part, but not posteriorly (H, I). In a partially mutant egg chamber (D), *puc* expression is present in wild-type cells only (E, F), indicating a cell autonomous requirement of *hep* (compare with wild-type expression of *puc* shown in C; see also M–R). Note the nonelongated appendages in the completely *hep* mutant chamber (I). puc^{B48} expression overlaps puc^{E69} expression in stretched cells (J). It is also expressed in border, centripetal and other columnar follicle cells (J–L). (L) A double staining revealing BR-C (blue) and puc^{B48} (red) overlapping patterns in the stretched and appendage cells. Anti-β-galactosidase stainings of *hep* mutant clones indicate that puc^{B48} is controlled by *hep* in all follicle cells (M–R), except the more posterior ones (not shown), as puc^{E69} (H, I). (D–I) Stage-13 to -14 egg chambers. (J, K–R) Stage-10 egg chambers. Anterior is to the left.

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rant patterning or morphogenesis. For example, mutations in the *BR-C* gene affect the determination of cells of the DA and lead to a phenotype that is very similar to that of *hep* (Deng and Bownes, 1997). In order to discriminate between these two possibilities, we examined pattern formation in the dorsal follicle cells earlier in oogenesis (stages 6–11). Once the nucleus has reached the dorsal–anterior corner of the oocyte, the associated Grk protein signals to the overlaying follicle cells for their determination. Activation of the EGFR is dynamic, being first induced widely in the dorsal region, as reflected by *kek* expression (Figs. 3B and 3C; Ghiglione *et al.,* 1999; Musacchio and Perrimon, 1996; Sapir *et al.,* 1998), and then becoming refined into two symmetrical patches of approximately 50 cells each, as reflected by *BR-C* expression (see Figs. 3E and 3H; Deng and Bownes, 1997). The expression pattern of *BR-C,* which integrates both AP and DV positional information, recapitulates the inductive history of the dorsal cells, and thus serves as an excellent marker for the determination of the follicle cells that will ultimately form the DA (Deng and Bownes, 1997). In the wild type, the BR-C protein is first expressed ubiquitously from stages 6 to 10. At stage 10a, BR-C expression is abolished in the dorsal–anterior-most region, facing the oocyte nucleus, and diminishes in the posterior and ventral regions. At stage 11, only two groups of dorsal follicle cells, which correspond to the progenitors of the DA, express BR-C. To establish whether *hep* is required for the determination of these cells, clones of a *hep* null allele were generated by using the UAS-FLP method (see Materials and Methods; Duffy *et al.,* 1998). We found that *hep* mutant clones located in the anterior–dorsal epithelium express *kek* (Figs. 3B and 3C) and *BR-C* normally (Figs. 3E, 3F, 3H, and 3I), indicating that the progenitors of the DA are normally formed and correctly positioned within the epithelium. These data are consistent with the JNK mutant phenotype, and suggest that the reduction in appendages size is not contributed by a loss or misdetermination of precursor cells, but rather by an abnormal subsequent morphogenesis.

These results show that both early and late activity of the EGFR pathway and BR-C expression are not affected by *hep*, and indicate that the *hep/DJNKK* pathway is not involved in the ERK-dependent patterning of the follicle cells. Since egg development is normal until stage 11, these observations suggest that the JNK cascade is a late-acting signaling pathway linking pattern formation to morphogenesis.

Hemipterous Controls puckered Expression in the Follicle Cells

The activity of the JNK pathway, and the extent of morphogenesis, in embryos and pupae have been shown to be controlled by a negative feedback loop involving the PUC MAPK phosphatase (Martin-Blanco *et al.,* 1998). In these tissues, *puc* expression is controlled by *hep* (Agnès *et al.,* 1999; Glise *et al.,* 1995; Zeitlinger and Bohmann, 1999) and can be ectopically induced by expressing activated forms of the small GTPases Drac1 and Dcdc42 (Glise and Noselli, 1997). Interestingly, *puc* is also expressed during oogenesis in the follicular epithelium, as shown by the expression pattern of the puc^{E69}–*lacZ* (Ring and Martinez Arias, 1993) and puc^{B48}–*lacZ* enhancer-trap lines (Fig. 4). The two lines show overlapping expression patterns. puc^{E69} expression is first detected at stages 10–11 of oogenesis in anterior stretched cells where it persists until stage 14 (Figs. 4A–4C). Expression is also found in the posterior part of some late stage-13 and -14 egg chambers (Figs. 4C and 4H). puc^{B48} is expressed in the main body follicle cells from stage 7 to stages 12–13. Expression then becomes more restricted to appendage precursors (overlapping with BR-C expression; Fig. 4L), border cells, centripetal cells, stretched cells, and posterior follicle cells (Figs. 4J–4L). These observations suggest a role for *puc* in cells that are precursors of DA and the micropyle, and prompted us to test for a potential regulatory role of *hep* on *puc* expression.

Egg chambers were stained with X-Gal and an anti-β-galactosidase antibody to detect *puc-lacZ* activity. In *hep* mutant clones, puc^{E69} and puc^{B48} expression is abolished or strongly reduced in stretched and main body follicle cells, indicating a requirement of the JNK pathway for *puc* expression in the follicular epithelium (Figs. 4D–4I and 4M–4R). In mosaic chambers, the loss of *puc* expression is restricted to the mutant cells (Figs. 4F, 4O, and 4R). This is the first demonstration that *hep* controls *puc* expression in a cell-autonomous manner. Some, but not all, stage-13 to -14 egg chambers also show expression in the posterior part of the egg chamber (Figs. 4C and 4H). As shown in a chamber that is completely mutant for *hep* (Figs. 4H and 4I), this posterior expression is not dependent on the function of *hep.* A *hep*-independent expression of puc^{E69} is also observed in other tissues and developmental stages (Agnès *et al.,* 1999). Together, these data indicate that *puc* expression is under the control of *hep* in all follicle cells, except for the more posterior cells in late stages.

Overexpression of puc Impairs DA and Micropyle Morphogenesis

puc encodes a MAPK phosphatase which is known to inhibit JNK pathway activity in different tissues (Agnès *et al.,* 1999; Glise *et al.,* 1995; Martin-Blanco *et al.,* 1998; Zeitlinger and Bohmann, 1999), and is thought to work in a negative feedback loop to modulate morphogenesis and stop cell sheet movements at the end of dorsal and imaginal discs closure. Consistent with this interpretation, overexpression of *puc* in the ectoderm during embryogenesis can mimic a loss of *hep* or *DJNK/basket* function (Martin-Blanco *et al.,* 1998). In order to test whether *puc* may have such a role during oogenesis, we used the UAS-GAL4 system to overexpress *puc* in different regions of the follicular epithelium.

Overexpression of *puc* in the posterior region (using the *E4GAL4* line; Queenan *et al.,* 1997) of the follicular epithelium has no effect on egg morphology and the embryo (data

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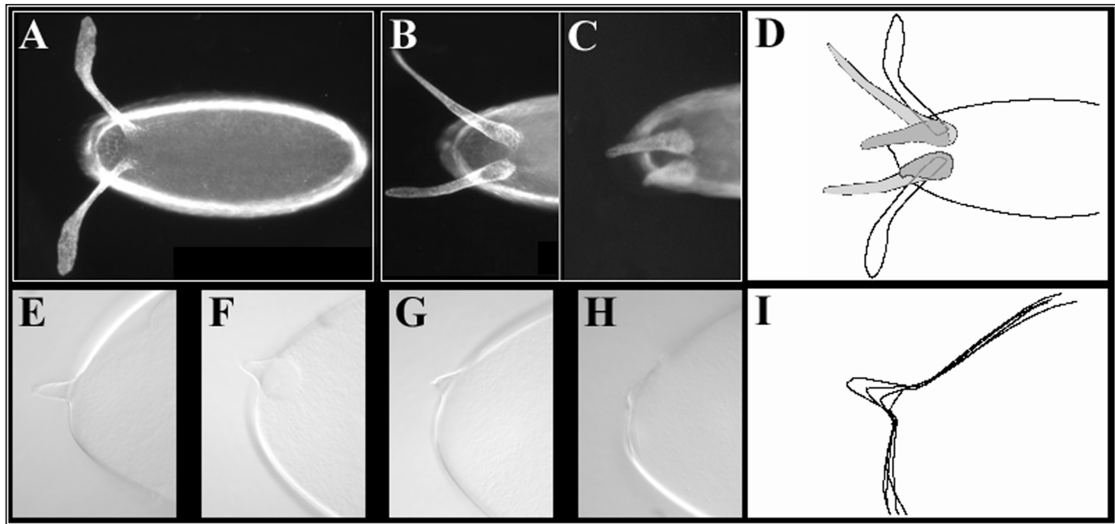

FIG. 5. *puc* overexpression affects the morphogenesis of the DA and micropyle. Dark field view of eggs laid by wild-type (A), *55BGAL4;*UAS-*puc* (B, C) females. (D) A superposition of (A–C) showing the differences in morphology of the appendages. When expressed anteriorly, *puc* reduces the extent of appendage elongation in a graded series of phenotypes. In most eggs, the main defect is an absence of the formation of the paddle-like structure that is characteristic of wild-type eggs; instead, appendages adopt a "tooth-pick"-like shape with pointed ends and a thickening of their bases. Normarsky views of the anterior part of dechorionated *E4GAL4;*UAS-*puc* (E, similar to wild type; data not shown) or *55BGAL4;*UAS-*puc* derived eggs (F–H). When expressed using the anterior *55BGal4* line, a vast majority of micropyles are very reduced in size. (I) A projection of the micropyles shown in (E–H) to illustrate the extent of the reductions. In the most severe cases (as in H), the micropyle has only approximately 25% the normal size. Anterior is to the left.

not shown). In contrast, expression of UAS-*puc* using *55BGAL4*, which drives expression in the anterior region of the main body follicle cells, induces a significant alteration in DA shape and size (85%, $n = 333$ at 29°C; Figs. 5B and 5C). A specific role for *puc* in follicle cells was confirmed by using two other GAL4 lines, T155 (97%, $n = 507$ at 29°C) and CY2 (data not shown), which drive strong expression in the entire columnar epithelium (Queenan *et al.,* 1997). The phenotypes ranged from slightly (Fig. 5B) to strongly reduced appendages (Fig. 5C). The characteristic bending of the distal part of the DA, the "paddle," is severely impaired, leading to "tooth-pick"-like straight appendages with pointed ends. These abnormal appendages, which are arrested later during their elongation than *hep*, *bsk*, and *Djun* mutant appendages (compare Fig. 5B and Fig. 1C, Figs. 2A and 2B), suggest that the formation of the paddle structure is a late event, taking place after full elongation. As a result of incomplete elongation, appendages show a typical enlargement of their base (Fig. 5D), as seen in *hep*, *bsk*, and *Djun* mutant eggs (Figs. 1 and 2).

In addition to the appendage defects, we found that a large proportion of eggs laid by 55B/UAS-*puc*, CY2/UAS-*puc*, or T155/UAS-*puc*, but not E4/UAS-*puc* females, showed a reduction in micropyle size (Figs. 5E–5H). GAL4 lines leading to a reduced micropyle are commonly expressed in the anterior part of the main body follicle cells. In order to better identify the cells that lead to micropyle defects when JNK activity is reduced, we expressed the *puc* gene under the control of the *slow border cells/slboGAL4* line. *slboGAL4* is expressed in two follicle cell populations (Rorth *et al.,* 1998), the border cells, which will form the micropyle canal (Montell *et al.,* 1992), and the centripetal cells, which will close the oocyte by stage 10 onward and participate in micropyle formation. Using this line, we were able to generate eggs with strongly shortened micropyles (99%, $n = 605$ at 29°C; Figs. 6B and 6C) but normal appendages, indicating that the JNK pathway is required in one or both of *slboGAL4* expression domains for correct micropyle morphogenesis. Since the border cells will contribute to the formation of the micropyle canal, and their loss does not affect micropyle size (Montell *et al.,* 1992), we conclude that the micropyle phenotype of JNK pathway mutants derives from aberrant centripetal cells morphogenesis.

Together, these results show that *puc* has a negative effect on DA and micropyle elongation and morphogenesis, when overexpressed in specific subsets of follicle cells, supporting an inhibitory role of *puc* in JNK signaling during oogenesis.

Micropyle Defects Are Not Due to Aberrant Border or Centripetal Cells Migration, nor to dpp Misregulation

During oogenesis, loss of *dpp* activity leads to anteriorly shifted appendages, while excess of *dpp* positions the appendages more posteriorly, indicating a role for the *dpp*

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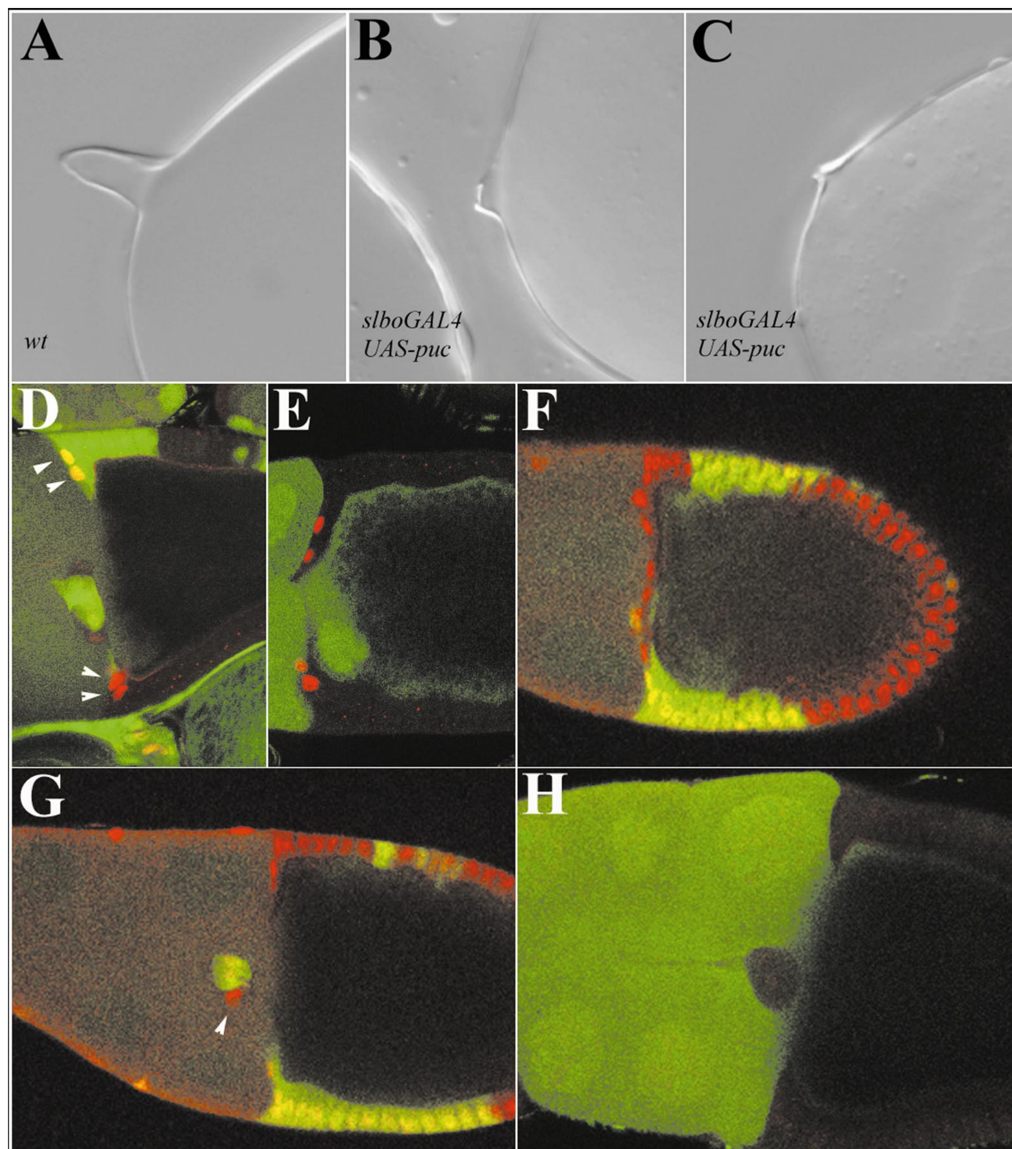

FIG. 6. Micropyle defects are not due to aberrant border or centripetal cells migration, nor to *dpp* misregulation. Nomarski views of micropyles derived from wild type (w^{1118}; A) or *slboGAL4;*UAS-*puc* flies (B, C). Using the *slboGAL4* line, expression is restricted to the border and centripetal cells (data not shown; Rorth *et al.,* 1998). This leads to a strong reduction of micropyles, without affecting the DA (not shown), indicating that defective JNK signaling in centripetal cells affects normal micropyle morphogenesis. (D–H) Confocal images showing the expression of *dpp* and *Dfos* in *hep* mutant follicle cell clones. *hep* mutant follicle cell clones are generated by using the FRT/UAS-FLP method (see Materials and Methods; Duffy *et al.,* 1998) with the following flies: *y w* hep^{r75} *FRT101/UB-GFP FRT101; e22cGAL4, UAS-FLP/*dpp^{10638}*-lacZ*. Mutant clones are marked by the absence of GFP. Ovaries were stained with either anti-β-galactosidase (to reveal *dpp-lacZ* expression; D, E) or anti-Dfos antibodies (which mark all follicle cells; F, G). *dpp* expression is normal in the leading edge of centripetal migrating cells. An asymmetrical clone in an early stage-10B egg chamber shows no differences between heterozygous and homozygous *hep* mutant cells expressing *dpp-lacZ* (D, arrowheads). An older stage-10B egg chamber that is completely mutant for *hep* in the follicle cells shows normal expression, ruling out any nonautonomous effect of *hep* on *dpp* expression. The *hep* micropyle phenotype cannot be attributed to aberrant cell migration of either centripetal (F, G) or border cells (D, G, H). (G) A *hep* mutant cell migrates normally with other heterozygous border cells, as does a fully mutant border cells clone. Anterior is to the left.

pathway in the correct patterning and positioning of the DA (Twombly *et al.,* 1996). Since the positioning of the appendages along the AP axis is not affected in JNK mutant eggs (Figs. 1, 2, and 5), it suggests that the JNK pathway does not affect *dpp* function in AP patterning. Interestingly, loss of *dpp* function can also lead to a reduction of the micropyle,

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though the origin of this phenotype is unknown (Twombly *et al.,* 1996). Relevant to these phenotypes, *dpp* is expressed in the stretched follicle cells and in a subset of cells making the leading edge of centripetal cells during their migration. The *dpp* expression domains thus overlap with domains of JNK activity. Since the JNK pathway controls *dpp* expression during dorsal closure (Glise and Noselli, 1997; Hou *et al.,* 1997; Riesgo-Escovar and Hafen, 1997b), we tested whether a subset of *dpp*-expressing cells could be controlled by *hep* during oogenesis. In *hep* mutant clones, both the levels and the pattern of *dpp-lacZ* expression (detected using *dpp*[10638], a *dpp-lacZ* enhancer-trap that recapitulates *dpp* endogenous expression in ovaries; Twombly *et al.,* 1996) are normal, as shown in the leading edge of centripetal cells (Figs. 6D and 6E). This result indicates that, despite a common micropyle phenotype, *dpp* expression is not coupled to JNK activity in ovaries, as it is in embryos.

In parallel, we also studied the migration of border and centripetal cells in *hep* mutant egg chambers, as a possible cause of micropyle abnormalities. The centripetal (Fig. 6F) and border cells migrate normally in chambers that have either partially (Fig. 6G) or completely (Fig. 6H) mutant follicle cells. These data suggest that the micropyle phenotype that is observed in *hep*, *bsk*, and *Djun* mutant eggs, as well as in *puc* overepression experiments, originates from a late defect in egg morphogenesis.

DISCUSSION

The making of a mature egg is a multistep process during which the oocyte differentiates, grows, acquires polarity, and is finally embedded into a shell secreted by the overlaying follicle cells (Spradling, 1993). During this maturation process, the activities of several signaling cascades are required and coordinated, with some of them, like the EGFR pathway, being used reiteratively (Ray and Schupbach, 1996). In this study, we show that the JNK pathway is required during late oogenesis for the morphogenesis of the DA and micropyle, thus adding a new player in the signaling machinery underlying egg formation. Since the other two MAPK pathways (ERK and p38) have also been shown to be involved in oogenesis (Ray and Schupbach, 1996; Suzanne *et al.,* 1999), the *Drosophila* egg chamber represents a paradigm for the study of multiple MAPK signaling pathways during development.

JNK Signaling and Chorion Morphogenesis

The outer follicular epithelium surrounding each oocyte secretes the chorionic envelope to protect the mature egg from external aggressions (Dobens and Raftery, 2000). During the late stages of its development, the follicular epithelium undergoes extensive morphogenesis in its anterior region, resulting in the decoration of the egg with few stereotyped structures. These include the DA, the operculum, and the micropyle, which are all essential for the egg. The micropyle allows sperm entry and fertilization, the operculum provides an exit for the hatching larvae, and the two DA serve as floating and breathing devices when the egg is covered by liquid. Interestingly, the DA show an extreme variation in their shape and number in different *Drosophila* species and recent work by Wasserman and Freeman (1998) show that the EGFR pathway may provide the molecular basis for this variability (Perrimon and Duffy, 1998; Wasserman and Freeman, 1998).

The analysis of *hep*, *bsk*, and *Djun* mutant clones indicates that JNK pathway activity is crucial in the follicular epithelium for DA morphogenesis. The observation of a complete series of phenotypes, ranging from reduced, "paddle-less" to completely nonelongated appendages, suggests that the JNK pathway plays a role in the elongation and shaping of these structures. As shown by the normal expression of two targets of the ERK pathway, *kek* and *BR-C* (Deng and Bownes, 1997; Ghiglione *et al.,* 1999; Musacchio and Perrimon, 1996; Sapir *et al.,* 1998), *hep* does not affect the patterning or development of the appendages during early and midoogenesis. We propose that the JNK pathway plays a previously unknown role in late oogenesis for appropriate morphogenesis of the DA and micropyle (Fig. 7). The unique phenotype of JNK pathway mutants may thus provide a link between pattern formation and morphogenesis in the egg chamber.

Our results suggest that *hep* and the JNK pathway lie downstream of both the EGFR and Dpp pathways in DA formation during late oogenesis. One interesting question is whether or not JNK activation is directly mediated by the ERK and/or Dpp pathways. Since both the ERK and JNK pathways are required for appendage formation, it is tempting to speculate that they may converge and their inputs integrate at the molecular level. One good candidate for such an integrating element is the AP-1 (activating protein-1) transcription factor, whose levels of expression and activity are regulated by both the ERK and JNK pathways in vertebrate cells (Karin *et al.,* 1997). As their vertebrate counterparts, the *Drosophila* Djun and Dfos homologs are also part of the JNK pathway (Kockel *et al.,* 1997; Riesgo-Escovar and Hafen, 1997a; Zeitlinger *et al.,* 1997), and these factors may also interact with the ERK cascade in the eye (Bohmann *et al.,* 1994; Peverali *et al.,* 1996; Treier *et al.,* 1995). Although the level of Dfos protein is normal in *hep* mutant follicle cells (Figs. 6F and 6G, and data not shown), analyzing the pattern of AP-1 activation in the egg chamber will be of particular interest to understand the relative contributions of the two MAPK pathways to appendage morphogenesis.

It has been observed that ectopic ERK activation in the posterior region of the egg can induce the formation of appendage-like material. However, this material does not fully elongate as normal appendages do, but remains very rudimentary, as observed in *hep, bsk* and *Djun* mutant clones (Peri and Roth, 2000; Queenan *et al.,* 1997; Spradling, 1993). A possible explanation for this "incompetence" to normally elongate is that the JNK pathway may not be

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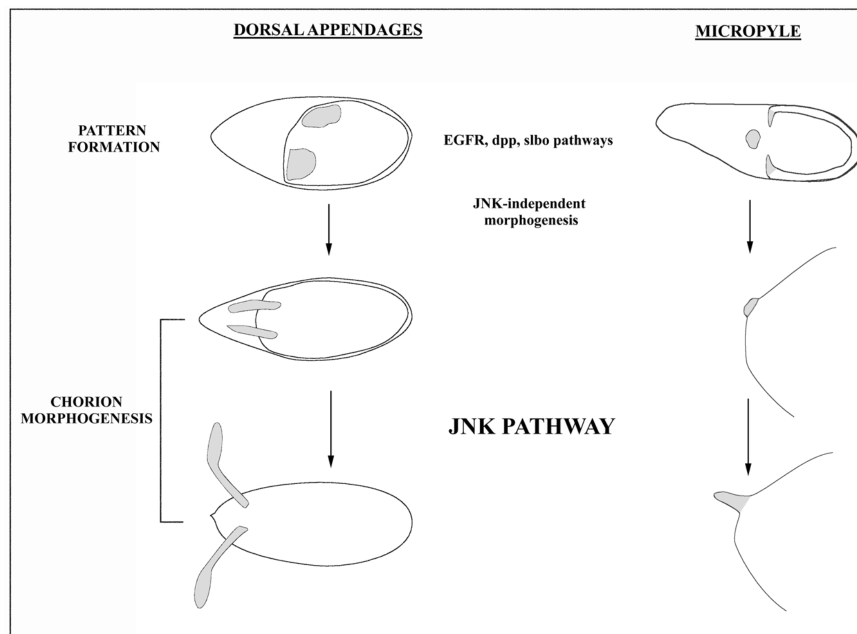

FIG. 7. Role of the JNK pathway in DA and micropyle morphogenesis. Different steps in DA and micropyle morphogenesis are schematized. The JNK pathway does not affect oogenesis untill stage 11, when DA start to elongate. For example, nonelongated appendages are visible in *hep* mutant stage-13 egg chambers (see Fig. 1G and Figs. 4D–4I). During micropyle morphogenesis, early stages are not affected either, as indicated by normal border and centripetal cells migrations (see Fig. 6). Based on JNK mutant phenotypes, we propose that DA and micropyle morphogenesis and elongation require both early JNK-independent and late JNK-dependent functions. For clarity, the germ cells and strectched follicular cells are not represented in late egg chamber drawings.

activated or fully inducible in the posterior part of the egg, due to the lack of some component(s) of the JNK pathway. In this respect, it is worth noting that *puc* expression escapes *hep* control in the posterior part of the follicular epithelium (Fig. 4H).

The *hep* chorionic phenotype is accompanied by a loss of *puc* expression in the anterior stretched and main body follicle cells, suggesting that these cells are important for JNK-dependent morphogenesis of the appendages. This is supported by overexpression of *puc* in subsets of columnar follicle cells. The use of a *slboGAL4* line allows to exclude a role for centripetal cells in DA formation (Fig. 6). These observations suggest that anterior main body follicle cells, including appendage precursor cells, and stretched cells, require *hep* function for DA elongation. Morphogenesis of the DA may thus require JNK activity in the two adjacent epithelia (stretched and columnar). For micropyle formation, the use of a *slboGAL4* line identified the centripetal cells as the ones requiring JNK activity for normal micropyle development. The absence of any obvious migratory defect in mutant border and centripetal cells exclude that micropyle shape defects are due to an early aberrant behavior of these two cell types. As for the DA, we propose that the micropyle is assembled in two steps: a *hep*-independent step requiring border and centripetal cells early migration, and a *hep*-dependent step that takes place during late stages (stage 11 onward) of oogenesis (Fig. 7).

Epithelial Morphogenesis and JNK Signaling

Epithelia are components of many different tissues, which they shape and make functional through elaborate morphogenesis. Different cellular mechanisms underlie the movement of epithelia, including folding (gastrulation), branching (tracheal development), or migration of entire sheets (dorsal closure, imaginal discs morphogenesis, wound-healing) (Goberdhan and Wilson, 1998; Noselli, 1998; Noselli and Agnes, 1999). One important goal is to identify the molecular mechanisms underlying these different behaviors, and understand how these are modulated to contribute to the diversity encountered in developing animals. One way to understand the basis of cell movement diversity is to compare related processes controlled by a single signaling cascade, like the JNK pathway. The comparison of dorsal closure and imaginal disc morphogenesis, which both are controlled by the JNK pathway in flies, allowed to propose a model for the morphogenesis of symmetrical epithelia containing "free margins" (Agnès *et al.,* 1999; Trinkaus, 1969). In this model, the morphogenesis or movement of bilateral epithelial sheets, like those taking place during dorsal closure, is driven by the activation of

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the JNK pathway in particular sites called margins. Interestingly, these margins are morphologically distinguishable, delineating two adjacent populations of cells: a columnar epithelium and a stretched one. In flies, several tissues show such an organization, including the lateral ectoderm in embryos and the imaginal discs. Strikingly, JNK activity in the egg chamber is essential for structures originating near such a boundary between a columnar (the main body or centripetal follicular epithelium) and a squamous epithelium (stretched cells), and may share several features with apparently different morphogenetic processes, like dorsal and thorax closures. Based on our current understanding of the JNK pathway in *Drosophila*, it is also tempting to speculate that every epithelium showing a discontinuity (i.e., the juxtaposition of a columnar and a stretched epithelium) may use the JNK pathway for its morphogenesis.

All the processes involving the JNK pathway also require a normal *dpp* activity, suggesting that these two pathways are intimately linked during epithelial morphogenesis in flies. During dorsal closure, but not during thorax closure (Agnès *et al.,* 1999), the JNK pathway controls the expression of *dpp* in leading edge cells (Glise and Noselli, 1997; Hou *et al.,* 1997; Riesgo-Escovar and Hafen, 1997b). Interestingly, we show that, during oogenesis, *dpp* expression is not under the control of the JNK pathway, as it is during dorsal closure. Thus, based on the presence or the absence of a transcriptional coupling between JNK and *dpp*, it is possible to define two different types of epithelial morphogenesis. In this respect, the way the JNK and *dpp* pathways are set up in the ovary is more similar to the situation found in imaginal discs. The study of JNK signaling in these different but related processes in *Drosophila* thus represents a unique system to study the molecular origin of diversity in epithelial morphogenesis.

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