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Mosaic analyses reveal the function of *Drosophila Ras* in embryonic dorsoventral patterning and dorsal follicle cell morphogenesis

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SUMMARY

In *Drosophila melanogaster*, the Ras signal transduction pathway is the primary effector of receptor tyrosine kinases, which govern diverse developmental programs. During oogenesis, epidermal growth factor receptor signaling through the Ras pathway patterns the somatic follicular epithelium, establishing the dorsoventral asymmetry of eggshell and embryo. Analysis of follicle cell clones homozygous for a null allele of *Ras* demonstrates that *Ras* is required cell-autonomously to repress *pipe* transcription, the critical first step in embryonic dorsoventral patterning. The effects of aberrant *pipe* expression in *Ras* mosaic egg chambers can be ameliorated, however, by post-*pipe* patterning events, which salvage normal dorsoventral polarity in most embryos derived from egg chambers with dorsal *Ras* clones. The patterned follicular epithelium also determines the final shape of the eggshell, including the dorsal respiratory appendages, which are formed by the migration of two dorsolateral follicle cell populations. Confocal analyses of mosaic egg chambers demonstrate that *Ras* is required both cell- and non cell-autonomously for morphogenetic behaviors characteristic of dorsal follicle cell migration, and reveal a novel, *Ras*-dependent pattern of basal E-cadherin localization in dorsal midline follicle cells.

Key words: *Drosophila melanogaster*, Dorsal-ventral polarity, Follicle cell, Cell migration, Epithelial morphogenesis, Oogenesis, *Ras*, *Egfr*, *pipe*, E-cadherin

INTRODUCTION

Ras-mediated signal transduction is conserved among eukaryotes and governs a host of diverse cellular processes, both normal and diseased. Members of the growing superfamily of related GTPases, Ras subfamily proteins primarily function to activate the canonical MAP kinase signaling cassette, propagating receptor tyrosine kinase (RTK) signals from the cell membrane to the nucleus (for a review, see Campbell et al., 1998). In addition to regulating cell growth, differentiation and apoptosis, Ras signaling also governs key events during normal development in diverse organisms, including yeast and mammals. In humans, Ras plays a pivotal – and well-documented – role during carcinogenesis, making it an obvious target for anti-cancer drug design.

In *Drosophila melanogaster*, the Ras signaling cassette is the major effector of RTK signaling during development, transducing signals from the epidermal growth factor receptor (Egfr), fibroblast growth factor receptor (FGFR/Breathless), Torso, and Sevenless RTKs (for a review, see Wassarman et al., 1995). Sophisticated genetic experiments have revealed the developmental functions for RTK/Ras signaling, including terminal patterning, ventral ectoderm patterning, photoreceptor development and specification, cell growth, and tracheal branching (for reviews, see Schweitzer and Shilo, 1997; Wassarman et al., 1995). In the *Drosophila* ovary, *Ras* functions downstream of *Egfr* to induce dorsal cell fate in the follicular epithelium (Schnorr and Berg, 1996).

Dorsoventral patterning of the eggshell and embryo occurs during oogenesis and is accomplished by cell-cell communication between the somatic follicular epithelium and the underlying germline cells (for reviews, see Nilson and Schüpbach, 1999; Van Buskirk and Schüpbach, 1999). Dorsoventral symmetry is broken midway through oogenesis, when the oocyte nucleus (and associated *gurken* (*grk*) mRNA) migrates from its posterior location to a random position at the anterior cortex of the oocyte (Roth et al., 1999). This newly relocalized source of the germline ligand Gurken (TGFα) activates Egfr in overlying somatic follicle cells, establishing a dorsal fate that is revealed by asymmetric expression of target genes along the dorsoventral axis.

Dorsoventral patterning of the follicle cells produces dorsoventral asymmetry in two distinct structures: the eggshell, secreted by the follicle cells, and the embryo, which develops within the eggshell following oviposition. To pattern the eggshell, Gurken activates Egfr in the dorsal-most follicle cells, triggering an amplification and spatial refinement of Egfr activity that can be monitored by detecting the active form of MAP kinase (Peri et al., 1999). The genes *spitz*, *vein* and *rhomboid* function in the follicle cells to amplify dorsal Egfr activity; then *argos*, which encodes an Egfr inhibitory ligand,

downregulates Egfr activity on the dorsal midline, refining a single broad dorsal peak of Egfr activity into two dorsolateral peaks (Wasserman and Freeman, 1998). In addition to this elaborate dorsoventral patterning process, anterior Decapentaplegic (Dpp) signaling specifies the anterior-posterior dimensions of the two dorsolateral regions of peak Egfr activity (Peri and Roth, 2000). During the final stages of oogenesis, these two anterior, dorsolateral groups of cells undergo morphogenesis and secrete the chorion that constitutes the two dorsal respiratory appendages of the eggshell.

Like eggshell patterning, dorsoventral patterning of the embryo is initiated by dorsal Gurken signaling (for a review, see Anderson, 1998). Egfr activity in dorsal follicle cells of the egg chamber represses transcription of *pipe* (*pip*), which encodes a putative glycosaminoglycan-modifying enzyme that functions together with the products of the ubiquitously expressed genes *nudel* and *windbeutel* to initiate an extracellular proteolytic cascade that culminates in the activation of the Toll receptor in the embryonic plasma membrane. Ventral Toll signaling induces nuclear translocation of the NF-κB/Rel homolog Dorsal in a gradient: highest in ventral embryonic nuclei to undetectable in dorsal nuclei. This gradient of nuclear Dorsal determines the zones of expression for zygotic patterning genes along the dorsoventral axis (for a review, see Rusch and Levine, 1996). Importantly for this work, loss-of-function and ectopic-expression data indicate that *pipe* is both necessary and sufficient to induce embryonic ventral cell fates (Nilson and Schüpbach, 1998; Sen et al., 2000; Sen et al., 1998).

One outstanding mystery of dorsoventral patterning is that while *pipe* is expressed in only the ventral-most third of the follicular epithelium (Sen et al., 1998), the expression of genes activated by Gurken/Egfr signaling is detected in only the dorsal-most third of the epithelium (Ghiglione et al., 1999; Jordan et al., 2000; Reich et al., 1999; Ruohola-Baker et al., 1993). How, then, is *pipe* repressed in the middle, or lateral third of the epithelium? In one hypothesis, dorsal anterior Gurken/Egfr signaling induces secretion of a second morphogen that represses *pipe* at a distance (Jordan et al., 2000). Alternatively, Pai and colleagues (Pai et al., 2000) propose that Gurken directly defines the *pipe* repression domain.

Since eggshell and embryonic dorsoventral patterning are initiated by the same signaling event, they are phenotypically linked; thus, mutations in *gurken* or *Egfr* typically affect eggshell and embryonic structures equally (Clifford and Schüpbach, 1989; Nilson and Schüpbach, 1999; Schüpbach, 1987). Eggs laid by females transheterozygous for strong hypomorphic mutations in *Ras* exhibit eggshell defects similar to those laid by *gurken* or *Egfr* mutants, implicating Ras in dorsoventral patterning of the follicle cells (Schnorr and Berg, 1996). Surprisingly, however, these *Ras* hypomorphic mutations do not compromise embryonic dorsoventral patterning: *Ras* eggshells are ventralized but produce embryos with no detectable dorsoventral defects. This same differential phenotype is also seen with loss-of-function mutations affecting two other members of the *Drosophila* Ras signaling cassette, Raf and MEK (Brand and Perrimon, 1994; Hsu and Perrimon, 1994).

Two hypotheses can explain this discrepancy between eggshell and embryonic phenotypes resulting from *Ras* mutations. First, both eggshell and embryonic patterning absolutely require *Ras*, but eggshell patterning is more sensitive than embryonic patterning to reductions in Ras. Indeed, both *Ras* and *mek* mutations can suppress the dorsalized embryo phenotype resulting from mislocalization of *grk* mRNA in *K10* mutant females, revealing a role in embryonic patterning (Hsu and Perrimon, 1994; Schnorr and Berg, 1996). This hypothesis predicts that *Ras* null follicle cells would fail to repress *pipe*, leading to severe defects during embryonic dorsoventral patterning. Alternatively, an effector pathway other than the Ras signaling cassette relays Egfr activity to the nucleus to initiate dorsoventral patterning of the embryo. The EGF receptor can signal through Ras-independent effectors in mammalian cells, such as Src kinases, phospholipase Cγ and phosphatidylinositol 3-kinase (for a review, see Schlessinger, 2000), all of which have *Drosophila* homologs (Lu and Li, 1999; MacDougall et al., 1995; Shortridge and McKay, 1995). This hypothesis predicts that Ras^{null} follicle cells would exhibit normal dorsal *pipe* repression, resulting in mild, if any, dorsoventral patterning defects in the embryo.

Here, we test these hypotheses by generating *Ras* null follicle cell clones. These mosaic analyses reveal that *Ras* is required to repress *pipe* transcription, the critical first step in embryonic dorsoventral patterning. However, additional embryonic patterning events downstream of *pipe* can compensate for loss of *Ras* function in dorsal follicle cell clones, demonstrating that embryonic dorsoventral patterning is a self-regulating, robust process. Furthermore, dorsal *Ras* clones do not affect lateral repression of *pipe*, adding support to the hypothesis that low levels of Gurken repress *pipe* laterally rather than a secondary signal emanating from dorsal follicle cells. Additionally, we characterize the requirement for *Ras* in dorsal appendage morphogenesis. Confocal analyses of mosaic follicular epithelia reveal that *Ras* is required at the onset of dorsal follicle cell morphogenesis and for a novel basal localization pattern of the adhesion protein *E-cadherin* on the dorsal midline. We propose a hypothesis for dorsoventral patterning that explains the disparity between the eggshell and embryonic patterning phenotypes of *Ras* hypomorphic mutants and we address why those phenotypes are not shared by *Egfr* mutants. Finally, we discuss the potential roles for, and possible effectors of, Ras signaling in the morphogenesis of dorsal follicle cells.

MATERIALS AND METHODS

Follicle cell mosaics

We employed the FLP/FRT system of mitotic recombination (Xu and Rubin, 1993) to generate marked follicle cell clones homozygous for $Ras^{\Delta C40b}$, a null allele of *Ras oncogene at 85D* (formerly called *Ras1*) that deletes the open reading frame (Hou et al., 1995; Schnorr and Berg, 1996). To analyze dorsal appendage defects, we generated females of genotype *hsFLP; FRT82B* $Ras^{\Delta C40b}$*/FRT82B hsNmyc*, heat-shocked them at 37°C for 1 hour to induce mitotic recombination, placed them at 25°C in fresh vials with Canton-S or w^{1118} males, then transferred the flies to fresh, yeasted food daily for 5-7 days. To examine clones, we induced N-myc expression with a 1 hour, 37°C degree heat-shock followed by a 30-minute to 1-hour recovery, dissected ovaries, and stained for the myc epitope and other markers, when appropriate.

To ensure that the defects we detected were due to loss of *Ras* function and not a background mutation, we compared the eggshell phenotypes of two independent *FRT82B* $Ras^{\Delta C40b}$ chromosomes and found them to be identical (data not shown). To analyze wild-type *pipe-lacZ* expression, we dissected females of genotype *w; pipe-lacZ*. For all remaining experiments, we used *Ras* mosaic females of genotype *hsFLP/+; pipe-lacZ/+; FRT82B* $Ras^{\Delta C40b}$*/FRT82B hsNmyc*. As a negative control, we used females genetically identical to the *Ras* mosaic females except that they bore a wild-type *FRT82B* chromosome instead of the *FRT* $Ras^{\Delta C40b}$ chromosome. We refer to clones in egg chambers from these flies as 'control clones'. To ensure that the absence of the myc epitope in mosaic egg chambers was due to the presence of a clone rather than a mechanical insult to the epithelium, we co-stained egg chambers with DAPI (4′,6-diamidino-2-phenylindole) to detect the presence of nuclei and, hence, intact follicle cells (data not shown).

To generate clones homozygous for the null allele $Egfr^{CO}$ (Clifford and Schüpbach, 1989), we employed the same protocol as above, except the females were of genotype *hsFLP/+; FRT42D* $Egfr^{CO}$*/FRT42D hsπmyc; +/TM6, pipe-lacZ*. The *TM6, pipe-lacZ* strain was constructed by mobilizing a second-chromosome *P[*w^{+}*; pipe-lacZ]* element using Δ2-3 transposase and standard genetic crosses.

Frequency and size of marked follicle cell clones

By dissecting ovaries at multiple time-points after FLP induction, we found that 6 days was optimal to generate relatively large *Ras* clones of 57 cells, on average (*n*=30), in stage 9-12 egg chambers. This time-frame also produced the highest possible frequency of *Ras* clones and associated eggshell defects, with 20% of stage 9-12 (*n*=146) egg chambers containing a clone and 16% of stage 14 eggshells bearing dorsal appendage defects (*n*=158). By comparison, control clones were composed of 106 cells, on average, in stage 9-12 egg chambers at 6 days post-induction (*n*=39), and 24% of control egg chambers contained a clone (*n*=169), roughly consistent with previously reported results for wild-type clones (Margolis and Spradling, 1995). Only 1% of stage 14 control clone eggshells bore dorsal appendage defects (*n*=115).

The reduced average size and frequency of *Ras* clones compared to control clones indicates a cell division or survival deficit of the $Ras^{\Delta C40b}$ cells. Furthermore, by dissecting females 10 days after induction, we attempted to identify large clones derived from follicle cell stem cells homozygous for $Ras^{\Delta C40b}$. We were unable to recover such clones, suggesting a requirement for *Ras* in follicle cell stem cell division or survival, or, alternatively, in the continued survival of clones growing from $Ras^{\Delta C40b}$ stem cells.

While *Ras* clones were recovered at a relatively high frequency 5-7 days post-induction, $Egfr^{CO}$ clones were virtually nonexistent at those time points: at 5 days post-induction, only 2% of egg chambers bore $Egfr^{CO}$ clones (*n*=176) of an average size of 5 cells (*n*=4). By contrast, 60% of control egg chambers bore clones (*n*=85) of average size of 275 cells (*n*=18). By decreasing post-induction time to 3 days, we improved the frequency of $Egfr^{CO}$ clones to 30% (*n*=146), with an average clone size of 10 follicle cells (*n*=67). At this same time point, 88% of control egg chambers had clones of average size 16 cells (*n*=103). Since $Egfr^{CO}$ clones were smaller and less frequent than $Ras^{\Delta C40b}$ clones, we hypothesize that Egfr perdures for a shorter time or plays a different role than *Ras* in follicle cell division and/or survival.

Immunofluorescence and microscopy

Ovaries were fixed and stained according to the method of Jackson and Berg (Jackson and Berg, 1999), except that 0.2 μg/ml DAPI was added during the third post-secondary wash. The following antisera were used: rabbit anti-β-galactosidase (Cappel, 1:1000), mouse monoclonal anti-c-myc (Oncogene, 1:50), rat monoclonal anti-*Drosophila*-E-cadherin 'D-CAD2' (1:50) (Oda et al., 1994). Primary antibodies were detected using standard dilutions (1:100-1:500) of fluorescently labeled secondary antibodies from Molecular Probes except for the rabbit β-galactosidase antibody, which was detected using anti-rabbit-Texas Red (Cappel, 1:1000).

For anti-Twist immunofluorescence with embryos, we employed a standard antibody protocol (Protocol 95) (Ashburner, 1989) with the following modifications. Embryos were dechorionated in 50% bleach, fixed in PBS/50% heptane/2% paraformaldehyde, and, after devitellinization, stored in methanol at 4°C. Peroxide treatment was skipped, washes were done in PBT (PBS + 0.2% Tween 20), and blocking and antibody incubations were done in PBT/5% BSA/0.02% sodium azide. Embryos were incubated in 1:5000 anti-Twist primary antibody (Roth et al., 1989), rinsed in PBT, incubated in 1:2000 biotinylated anti-rabbit antibody, rinsed again, then incubated in 1:100 streptavidin-FITC conjugate.

Labeled ovaries and embryos were examined using a Biorad MRC600 confocal microscope. A Nikon Microphot-FXA was used for rapid scoring of mosaic egg chambers, detection of DAPI, and DIC microscopy. Images were analyzed and processed using NIH Image and Adobe Photoshop.

RESULTS

Ras and *Egfr* are required cell-autonomously in dorsal and lateral follicle cells for repression of a *pipe* transcriptional reporter

Unlike *gurken* and *Egfr* mutants, females transheterozygous for strong hypomorphic alleles of *Ras* lay ventralized eggs from which a high proportion of embryos hatch. Therefore, either eggshell patterning is more sensitive than embryonic patterning to reductions in Ras, or alternatively, Ras-independent effectors of Egfr may mediate embryonic patterning events. These hypotheses can be distinguished by examining embryonic patterning in egg chambers with follicle cells that completely lack *Ras* function. Since the absence of *Ras* is lethal, we generated follicle cell clones homozygous for the null allele $Ras^{\Delta C40b}$, then tested whether *Ras* clones affect embryonic dorsoventral patterning by analyzing a reporter for *pipe* expression in mosaic ovaries. *pipe* is an excellent marker because its function in follicle cells is both necessary and sufficient to induce ventral cell fate in the embryo (Nilson and Schüpbach, 1998; Sen et al., 1998). Moreover, its expression in follicle cells allows simultaneous detection of *pipe* and the marked clones. To test whether *Ras* is required for *pipe* repression, we analyzed expression of the promoter fusion construct *pipe-lacZ* in *Ras* mosaic egg chambers (Sen et al., 1998).

First, to ensure the accuracy of our analyses, we characterized how *pipe-lacZ* expression is spatially refined over time in wild-type egg chambers (Fig. 1) and then compared that *pipe-lacZ* pattern to the published *pipe* mRNA expression pattern (Sen et al., 1998) (F. Peri, M. Technau and S. Roth, personal communication). Beginning at stage 7 and until stage 10B, *pipe-lacZ* was expressed in posterior follicle cells and, as posterior follicle cell migration proceeded, in the nascent stretch follicle cells. Prior to stage 9 of oogenesis, *pipe-lacZ* was also expressed in a sagittal ring of follicle cells at the anterior margin of the growing columnar epithelium (Fig. 1A, bracket), and by stage 10B, only the ventral cells of the ring retained expression, constituting the anterior part of the well-defined stage 10 *pipe* pattern (Fig. 1D). Importantly, at the anterior of the columnar epithelium, *pipe-lacZ* expression

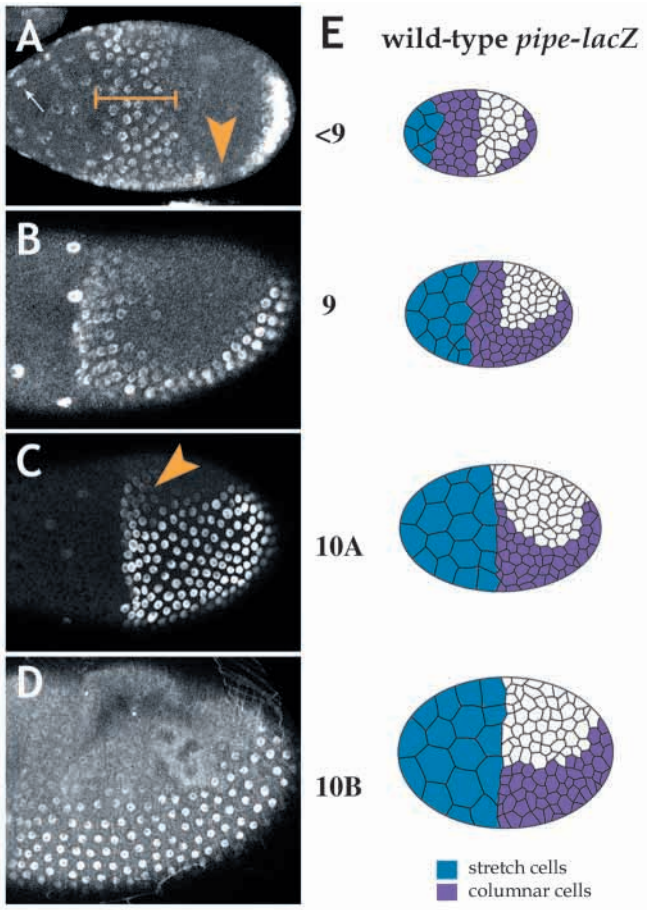

Fig. 1. Wild-type *pipe-lacZ* expression is dynamic during oogenesis. (A-D) Confocal projections of egg chambers from females homozygous for the *pipe-lacZ* reporter construct. *lacZ* expression was detected using an anti-β-gal antibody. Lateral views are shown, except where noted, with anterior to the left and dorsal at top. (A) At stage 8, *pipe-lacZ* is expressed in the anterior follicle cells that stretch to compensate for posterior migration (small white arrow) and in a sagittal ring (bracket) at the anterior margin of the posteriorly migrating group. Posterior follicle cells, and some posterior ventral follicle cells, express *pipe-lacZ*, with a gap in ventral expression about halfway across the columnar epithelium (arrowhead). (B) Dorsolateral view. As posterior follicle cell migration is completed at the end of stage 9, stretch cell and sagittal ring expression are maintained and ventral expression becomes contiguous. (C) Ventrolateral view. During stage 10A, the sagittal ring pattern gradually disappears, beginning dorsally and proceeding ventrally, such that expression protrudes dorsally from the anterior end of the ventral expression domain (arrowhead). (D) By stage 10B, dorsal sagittal ring expression has completely disappeared, while expression remains in the ventral, stretch (not shown), and posterior follicle cells. (E) Schematic diagram depicting wild-type *pipe-lacZ* expression in stretch (blue) and columnar (purple) follicle cells over time.

protruded dorsally from the ventral domain in many stage 10A and early 10B egg chambers (Fig. 1C, arrowhead). This protrusion is the last remnant of dorsal sagittal ring expression before it finally resolves into the ventral zone of expression. This observed *pipe-lacZ* expression is consistent with the reported pattern of endogenous *pipe* mRNA (Sen et al., 1998) (F. Peri, M. Technau and S. Roth, personal communication).

Next we analyzed *pipe-lacZ* expression in $Ras^{\Delta C40b}$ and control mosaic egg chambers. In a completely cell-autonomous manner, *pipe-lacZ* was ectopically expressed at high levels in 100% of dorsal (n=18) and lateral (n=8) $Ras^{\Delta C40b}$ follicle cell clones (Fig. 2B). Ventral *Ras* clones had no effect on *pipe-lacZ* expression (i.e., *pipe* remained on, data not shown). As expected, egg chambers bearing large control clones at dorsal and lateral positions had normal *pipe-lacZ* expression patterns (n=9, Fig. 2A). Although $Egfr^{CO}$ clones were smaller and less frequent than *Ras* clones (see Materials and Methods), they, too, displayed cell-autonomous *pipe-lacZ* derepression (Fig. 2C, n=17 dorsal and lateral clones). These data demonstrate that *Ras* and *Egfr* are required cell autonomously for *pipe-lacZ* repression in dorsal and lateral follicle cells. Since *pipe* is necessary and sufficient to induce ventral embryo fates, our data suggest that *Ras* is required in follicle cells to initiate dorsoventral patterning of the embryo.

Corroborating these results are recent experiments in which the dorsal repression of *pipe* mRNA is cell-autonomously relieved in *Raf* loss-of-function follicle cell clones (F. Peri, M. Technau and S. Roth, personal communication). Raf functions downstream of *Egfr* and *Ras* in dorsal follicle cells as part of the canonical Ras/MAPK signaling cassette and is required for dorsoventral patterning of the follicular epithelium (Brand and Perrimon, 1994).

Dorsal *Ras* clones do not affect lateral repression of *pipe*

To address whether lateral repression of *pipe* depends directly on Gurken or on a secondary signal emanating from the dorsal anterior follicle cells, we asked whether lateral *pipe-lacZ* repression was altered by the presence of dorsal *Ras* clones. We reasoned that if the secondary signaling hypothesis is correct, dorsal patches lacking *Ras* would not only lose repression of *pipe* in a cell-autonomous fashion, but would also fail to secrete secondary signaling molecules to repress *pipe* at a distance. In contrast, if lateral repression of *pipe* results from direct contact with low levels of Gurken at lateral positions in the oocyte, dorsal *Ras* clones should not compromise lateral repression of *pipe*.

In five of eight lateral views of egg chambers with dorsal *Ras* clones, lateral repression of *pipe-lacZ* appeared normal (Fig. 2D). Three of eight egg chambers, however, exhibited a mild dorsal expansion of the ventral *pipe-lacZ* expression domain (Fig. 2E), but these expansions did not differ significantly from those characteristic of the dynamic wild-type *pipe-lacZ* expression pattern (Fig. 1B,C). Therefore, the presence of dorsal *Ras* follicle cell clones did not strongly affect lateral repression of *pipe-lacZ*, supporting the hypothesis that Gurken directly represses *pipe* in lateral follicle cells. Consistent with these data, Peri and colleagues (F. Peri, M. Technau and S. Roth, personal communication) report similar results from examination of *pipe-lacZ* in marked loss-of-function *Raf* clones. Although *Raf* clones cause cell-autonomous de-repression of *pipe-lacZ*, dorsal *Raf* clones do not disrupt lateral *pipe-lacZ* repression. Furthermore, they show that clones expressing a constitutively activated form of the EGF receptor do not disrupt *pipe-lacZ* expression in surrounding cells.

Our observations notwithstanding, it is still possible that the wild-type unevenness of the lateral edge of *pipe-lacZ*

expression may have masked a subtle expansion in *pipe-lacZ* caused by dorsal *Ras* clones. Therefore, we conclude that if dorsal *Ras* clones relieve lateral *pipe-lacZ* repression, the effect is very small. Our result is consistent with the hypothesis proposed by Pai et al. (Pai et al., 2000), in which Gurken directly defines the *pipe* repression domain.

Dorsoventral patterning events downstream of *pipe* can compensate for loss of *Ras* function in dorsal follicle cell clones

Mosaic analyses demonstrate that loss of *pipe* in ventral follicle cells leads to local embryonic dorsalization (Nilson and Schüpbach, 1998). Furthermore, *pipe* expression is sufficient to induce ventral embryonic cell fate since ectopic expression of *pipe* throughout the follicular epithelium causes embryonic ventralization (Sen et al., 1998). These results point to a spatial relationship between *pipe* in the egg chamber and ventral cell fate in the embryo. Therefore, we predicted that the ectopic expression of *pipe* observed in *Ras* follicle cell clones should translate into local embryonic patterning defects.

Dorsoventral patterning of the embryo can be directly visualized in cellularizing embryos by detecting asymmetrically expressed molecular markers. High levels of nuclear Dorsal induce the expression of Twist protein in a ventral stripe occupying approximately 20% of the embryonic circumference, which invaginates at the onset of gastrulation to form the ventral furrow (Thisse et al., 1988). Mutations in *gurken* and *Egfr* ventralize the eggshell and embryo, expanding embryonic Twist protein dorsally (Roth and Schüpbach, 1994).

To test whether the ectopic expression of *pipe* observed in *Ras* follicle cell clones translates directly into ectopic Twist in the embryo, we examined Twist protein in cellularizing embryos derived from egg chambers bearing dorsal *Ras* clones. The study of embryonic phenotypes resulting from follicle cell clones is difficult because the follicular epithelium is sloughed off before embryonic development begins. Therefore, we took advantage of the fact that eggs laid by *Ras* mosaic females displayed dorsal appendage defects associated with dorsal anterior *Ras* clones (Figs 4, 5). By selecting eggs with appendage defects indicative of dorsal anterior clones (Fig. 4E, asterisks), we ensured analysis of Twist protein specifically in embryos resulting from egg chambers with dorsal *Ras* clones. We avoided ventralized eggs (see drawing in Fig. 4E) because that phenotype may also result from posterior *Ras* clones that prevent anterior-posterior patterning, a prerequisite for dorsoventral patterning. We predicted that, if ectopic *pipe* in the egg chamber is sufficient to induce ectopic Twist in the embryo on a cell-by-cell basis, 100% of eggs with appendage defects should display ectopic Twist expression in the dorsal anterior region of the cellularizing embryo.

Surprisingly, we detected ectopic Twist protein in only five of the 26 cellularizing embryos developing within eggshells bearing appendage defects (Fig. 3B-D). Most (21/26) embryos were indistinguishable from those derived from control clone eggs (Fig. 3A). Furthermore, in only two of the five affected embryos was the ectopic Twist expression in the dorsal anterior of the embryo (Fig. 3C,D); the remaining three embryos exhibited lateral or posterior expansions of the Twist domain (Fig. 3B). These eggs may have borne additional clones elsewhere in the follicular

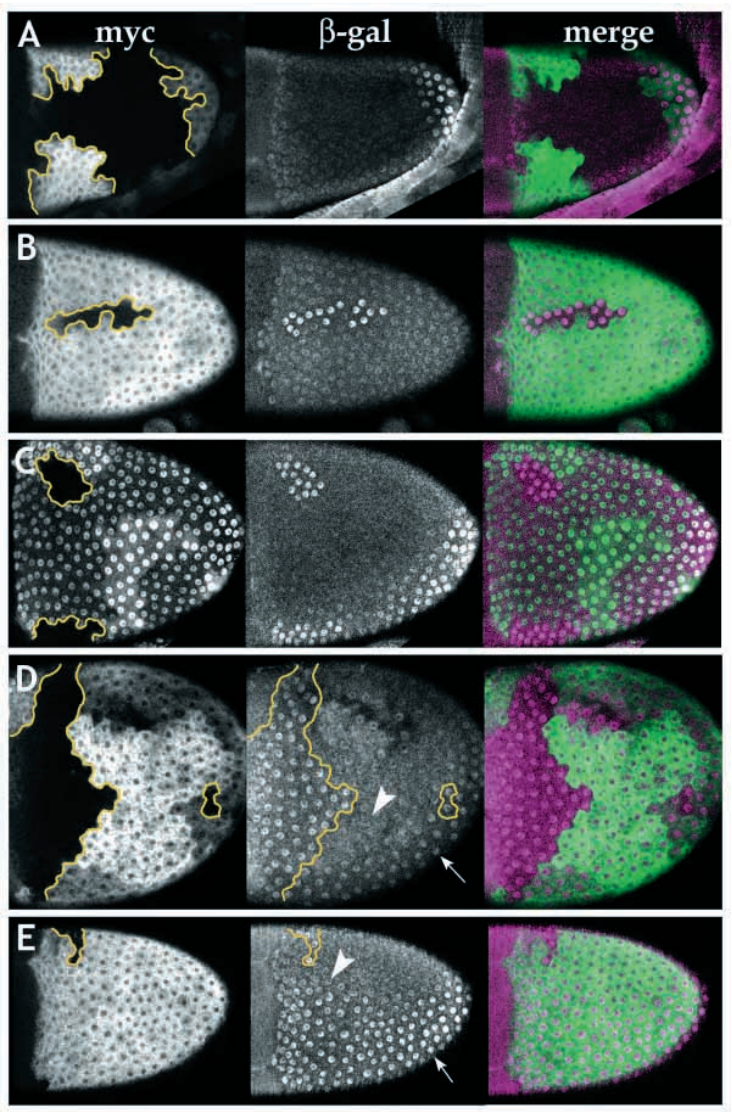

Fig. 2. *Ras* and *Egfr* are required cell-autonomously for dorsal repression of *pipe-lacZ*. Confocal projections of control (A), $Ras^{\Delta C40b}$ (B,D,E), and $Egfr^{CO}$ (C) follicle cell clones, marked by the absence of the myc epitope. Clones are outlined in yellow. *pipe-lacZ* expression was detected by β-gal antibody. Anterior is to the left in all panels. (A) Dorsal view of a large control clone in which dorsal *pipe-lacZ* repression is unaffected. The posterior expression seen in this egg chamber is normal and is contiguous with the normal ventral region of *pipe-lacZ* expression (out of the plane of focus). Dorsal appendages from an adjacent S14 egg chamber abut the posterior half of this egg chamber. (B) *pipe-lacZ* is cell-autonomously derepressed in a dorsal *Ras* follicle cell clone. (C) Dorsolateral view of two *Egfr* clones: dorsal clone (top) and ventrolateral clone (bottom) both cell-autonomously derepress *pipe-lacZ*. (D,E) Lateral views of *Ras* mosaic egg chambers. In both egg chambers, the edge of the normal ventral and posterior domains of *pipe-lacZ* expression can be seen (small arrows), as can cell-autonomous *pipe-lacZ* derepression in the clones. (D) A patch of lateral follicle cells ventral/posterior to a large dorsal/lateral *Ras* clone still represses *pipe-lacZ* (large arrowhead). (E) Ventral *pipe-lacZ* expression spreads dorsally (large arrowhead) into the lateral region in this egg chamber with a dorsal *Ras* clone. This expansion, however, is also seen in wild-type (Fig. 1).

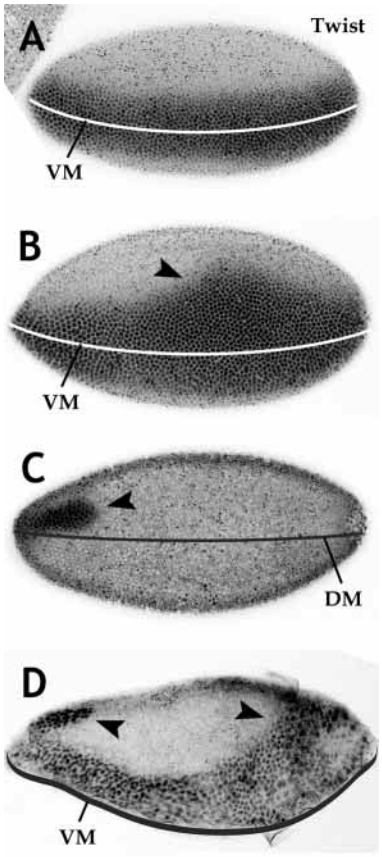

Fig. 3. Twist protein is normal in most embryos derived from egg chambers with dorsal *Ras* clones. (A-D) Confocal projections (black/white-inverted) of anti-Twist fluorescence in cellularizing embryos hand-picked based upon dorsal appendage phenotype. Anterior is to the left in all panels. VM, ventral midline; DM, dorsal midline. (A) Twist protein in an embryo derived from a control clone egg chamber. 21 of 26 embryos derived from egg chambers with dorsal *Ras* clones were indistinguishable from controls. (B-D) Three examples of embryos derived from egg chambers with dorsal *Ras* clones that did display ectopic Twist protein (arrowheads), which always contacted the normal ventral Twist domain.

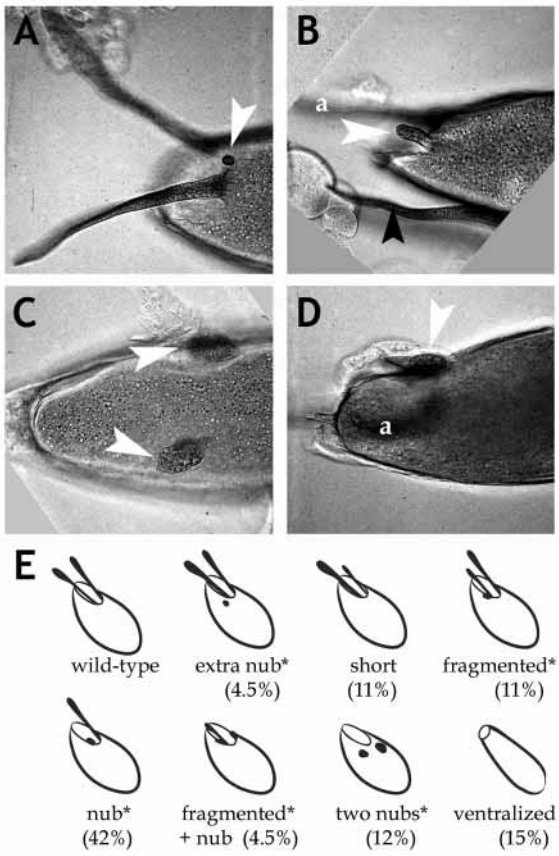

Fig. 4. Dorsal appendage phenotypes resulting from *Ras* follicle cell clones. (A) The 'extra nub' phenotype: eggshell has an extra nub of chorion (arrowhead) in addition to two wild-type dorsal appendages. (B) The 'nub' phenotype: a nub of chorion material (white arrowhead) replaces one of the dorsal appendages. The other phenotypically wild-type appendage (a) is out of the plane of focus. The appendage of a neighboring egg can also be seen (black arrowhead). (C) The 'two nubs' phenotype: nubs replace both appendages (arrowheads). (D) Some chorion nubs are shaped like a teardrop (arrowhead), with a bulbous appendage base tapering to an anterior point. The other phenotypically wild-type appendage is out of the plane of focus (a). (E) Drawings, with corresponding frequencies, of the various chorion phenotypes seen in late stage *Ras* mosaic egg chambers. Frequencies are calculated from the number of eggs displaying a particular phenotype divided by the total number of eggs with chorion defects (n=177). Phenotypes labeled with (*) were selected for embryonic analysis.

epithelium. Interestingly, the ectopic Twist detected in the five affected embryos always contacted the normal ventral stripe of Twist protein expression, suggesting that the *pipe* expression domain must exceed a certain minimum size in order to induce ectopic Twist. In the other 21 egg chambers, dorsal cells with ectopic ventral fate probably failed to induce ventral embryonic fate because they were surrounded on all sides by wild-type dorsal cells. To ensure that this particular set of crosses had indeed generated 'normal' *Ras* clones (e.g., *Ras* clones were present at the usual frequency and ectopically expressed *pipe*), we dissected ovaries from these females and confirmed ectopic *pipe-lacZ* expression in mosaic egg chambers (data not shown).

pipe-lacZ was ectopically expressed in 100% of *Ras* clones in a cell-autonomous manner (Fig. 2), but only two of the 26 embryos resulting from eggs with dorsal anterior *Ras* clones displayed dorsal anterior ectopic Twist. Similarly, *Raf* loss-of-function follicle cell clones rarely affect embryonic patterning (F. Peri, M. Technau and S. Roth, personal communication). These data suggest that additional patterning events downstream of *pipe* refine the initial asymmetry defined by dorsal Egfr signaling and establish the shape of the Dorsal gradient that determines embryonic fates along the dorsoventral axis.

Ras mutant cells fail to participate in dorsal follicle cell migration

Dorsoventral patterning within the follicular epithelium precedes the anterior migration of two dorsolateral populations of follicle cells, or dorsal appendage primordia, which ultimately secrete the dorsal respiratory appendages of the eggshell. Mutants carrying strong *Ras* loss-of-function mutations lay eggs with fused dorsal appendages, demonstrating the requirement for *Ras* during patterning of the

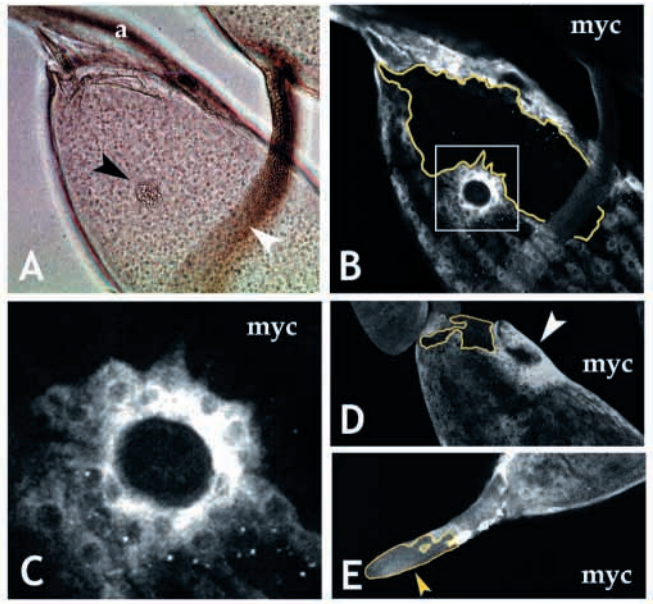

Fig. 5. *Ras* mutant cells do not participate in dorsal appendage formation. (A-D) Bright-field and confocal images of dissected stage 14 eggs with associated *Ras* follicle cell clones. Clones are identified by their lack of myc expression (outlined in yellow). (A) Chorion nub on a mosaic eggshell (black arrowhead). The other appendage on this egg (a) extends out of the plane of focus. An appendage from an adjacent egg also extends into the field (white arrowhead). (B) Confocal image of the egg chamber in A. A *Ras* clone covers a large portion of the dorsal anterior portion of the egg chamber, but the chorion nub is made by wild-type, myc^+ cells neighboring the *Ras* clone. (C) Enlargement of the box in B. The dark hole is filled with the chorion making up a nub, which was secreted by about 8 wild-type cells. Though not visible in this projection, these cells cover the entire nub. (D) Confocal projection of a mosaic egg chamber with 'teardrop'-shaped chorion nub. A *Ras* clone resides to the anterior of the group of wild-type cells making the nub, and appears to block the progress of the migrating cells. (E) Confocal projection of the anterior tip of a stage 14 control egg chamber showing that control clone cells are capable of migrating to the full anterior extent (arrowhead).

dorsal appendage primordia (Schnorr and Berg, 1996). Our mosaic analyses allowed us to ask whether *Ras* is also required for dorsal appendage morphogenesis and to examine the behavior of wild-type and mutant cells in mosaic egg chambers.

First, we evaluated the phenotype of eggs laid by *Ras* mosaic females 5-7 days after clone induction. 16% of stage 14 eggshells displayed some type of dorsal appendage defect (n=158). Defects ranged from the complete ablation of dorsal appendages in the most severe case, to a small 'nub' of extra chorion material adjacent to two normal dorsal appendages in the least severe case (Fig. 4). Ablation or, rarely, fusion of the dorsal appendages occurred only in a small fraction of defective eggs and likely resulted from either a very large dorsal anterior clone or a posterior clone that prevented posterior cell fate establishment and, therefore, hindered anterior migration of the oocyte nucleus, a prerequisite for dorsal Gurken signaling. The most common phenotype was a small 'nub' of chorion material in place of a normal dorsal appendage (Fig. 4B,D, Fig. 5A). Some defective eggs had two nubs in place of dorsal appendages (Fig. 4C); others bore shortened or fragmented dorsal appendages (Fig. 4E).

To determine whether aberrant nubs or fragments of dorsal appendages were formed by the *Ras* mutant cells or by wild-type (*Ras*/+ or +/+) cells adjacent to *Ras* clones, we dissected ovaries and analyzed stage 14 mosaic egg chambers with chorion defects (Fig. 5A-D). At this late stage, the follicular epithelium is rapidly degrading, but some stage 14 egg chambers can be found in which the follicular epithelium remains intact. In all nine of these egg chambers, extra chorion nubs or fragments of dorsal appendages were secreted by wild-type cells, and in all cases, these wild-type cells abutted *Ras* clones (Fig. 5B,D). Thus, *Ras* mutant cells did not migrate or secrete the appendage material forming the aberrant chorion structures, but rather, impinged on the activities of nearby wild-type cells, either by obstructing their movement or by reducing the number of cells fated to migrate. Interestingly, in one egg chamber, a nub of chorion material was surrounded by only about eight follicle cells, indicating that a relatively small number of wild-type cells can organize and secrete a dorsal appendage nub (Fig. 5B,C).

Some aberrant appendage structures had a characteristic 'teardrop' shape (Fig. 4D). These appendages form when wild-type cells at the posterior of a dorsal appendage primordium encounter a *Ras* clone in a more anterior position (Fig. 5D) and are unable to migrate between or over them. We speculate that the cells of the *Ras* clone adhere strongly to the substratum (basal lamina or stretch cells) and to each other, thereby blocking the anterior migration of the wild-type cells.

Finally, we searched stage 13 and 14 mosaic egg chambers for any mutant cells that might have been included, actively or passively, in the group of follicle cells extending anteriorly to make the dorsal appendages. In control mosaic egg chambers, clones were often found amongst the migrating population (Fig. 5E). In over 400 *Ras* mosaic egg chambers, however, we never detected mutant cells in the migrating group. Therefore, *Ras* mutant cells were neither found amongst the migrating cohort of dorsolateral follicle cells nor responsible for secreting aberrant chorion structures. Thus, *Ras* mutant cells failed to participate, actively or passively, in dorsal follicle cell migration or secretion of dorsal appendage chorion.

Ras mutant cells do not undergo cell shape changes characteristic of dorsal follicle cell morphogenesis

Analysis of late-stage mosaic egg chambers reveals the final outcome of dorsal follicle cell movements but does not show how wild-type and mutant cells behave throughout the morphogenetic process itself. We therefore investigated whether *Ras* mosaic egg chambers exhibit defects during the earliest events of dorsal appendage morphogenesis and how wild-type (*Ras*/+ or +/+) cells respond to neighbors lacking *Ras* function.

The two dorsolateral appendage primordia begin morphogenesis by undergoing a characteristic cell shape change in which apical diameter decreases relative to that of the cells outside the primordia (J. D., K. J., D. Kiehart and C. B., unpublished data). To test whether *Ras* mutant cells within the dorsal appendage primordia undergo this shape change, we carried out confocal analyses of mosaic egg chambers in which we simultaneously detected *Ras* clones and visualized cell morphology using one of two reagents: rhodamine-phalloidin,

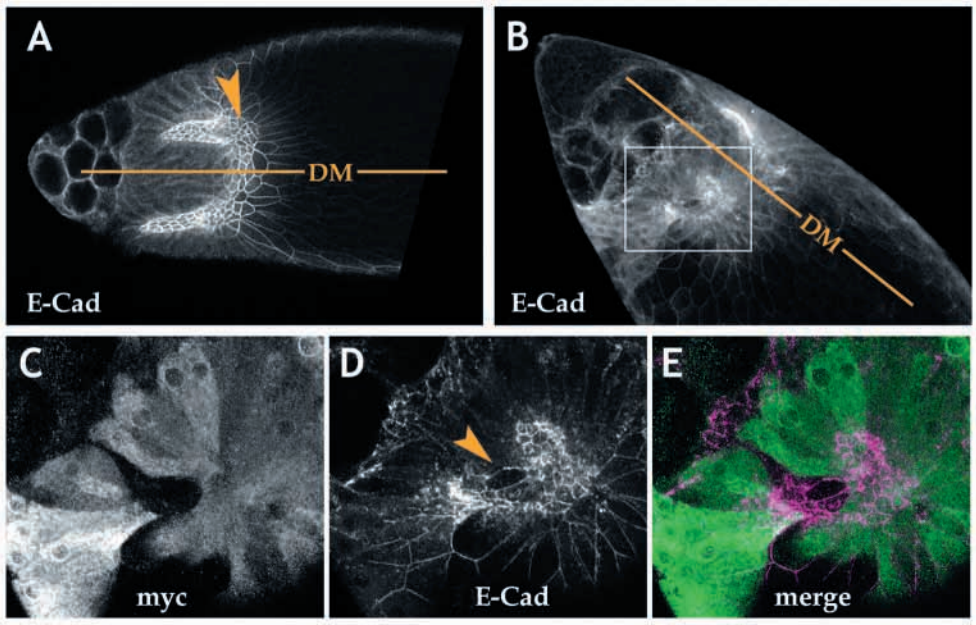

Fig. 6. *Ras* mutant cells within the dorsal appendage primordia fail to undergo a reduction in apical diameter. Confocal projections of apicolateral anti-E-cadherin (E-Cad) fluorescence in stage 12 egg chambers highlight the apical morphology of wild-type (A) and *Ras* mosaic (B) dorsal appendage primordia. DM, dorsal midline. (A) The bright apices of wild-type follicle cells undergoing dorsal appendage morphogenesis are uniformly small (arrowhead) unlike their neighbors, which have rather large apical surfaces. (B) Dorsolateral view. The left appendage primordium of a *Ras* mosaic egg chamber is disrupted. (C-E) Higher magnification views of the disrupted appendage primordium, boxed in B, reveal that *Ras* mutant cells – those lacking the myc epitope (C) – exhibit large apical footprints (D, arrowhead). Although the wild-type cells adjacent to the clone have small apical diameters, their overall organization is less uniform than expected.

which binds to filamentous actin, or anti-E-cadherin, which highlights apical morphology (Fig. 6A). Apical morphology of the dorsal appendage primordia was grossly disrupted in all egg chambers with dorsal anterior *Ras* clones (n=14). This disruption was particularly clear in one egg chamber in which a *Ras* clone covering an anterior region of the left appendage primordium displayed a marked increase in apical diameter (Fig. 6B-E). The apical diameters of the surrounding wild-type (*Ras*/+) cells in the primordium were appropriately small. We hypothesize that this egg chamber, if allowed to develop, would have produced a 'fragmented' dorsal appendage phenotype (see drawing in Fig. 4E) because the wild-type migrating cohort was split into two groups.

Other egg chambers with dorsal *Ras* clones exhibited similar defects; a general disruption of epithelial morphology, however, made it difficult to visualize specific effects on apical diameter. *Ras* mosaic epithelia contained bubbles and/or holes, unusual actin accumulations, and aberrant cell projections, and these disruptions occurred within *Ras* clones and also in wild-type cells bordering *Ras* clones (data not shown). Therefore, while the apices of the mutant cells appeared abnormally large, the overall epithelial disruptions preclude any strong conclusions regarding the specific requirement for *Ras* during this particular cell shape change. Nevertheless, our results clearly indicate that morphogenesis as a whole is disrupted at the time of this shape change. Since a reduction in apical diameter is one of the first events of dorsal follicle cell morphogenesis (J. D., K. J., D. Kiehart and C. B., unpublished data), our results suggest that *Ras* mutant cells fail to migrate because they fail to initiate morphogenesis. Furthermore, this failure to initiate morphogenesis can physically interfere with the movements of wild-type cells in the region by altering the overall morphogenetic architecture of a dorsal appendage primordium.

Ras is required for the basal localization of *E-cadherin* on the dorsal midline

Since *Ras* mutant follicle cells did not participate, not even passively, in dorsal follicle cell migration, and since these cells sometimes appear to obstruct the migration of wild-type follicle cells by adhering strongly to the substratum and preventing the anterior migration of the wild-type cells (Fig. 5D), we reasoned that *Ras* mutant cells may not properly regulate the expression or localization of adhesion molecules. Developmentally regulated adhesion mediated by *Drosophila* E-cadherin (E-Cad) orchestrates specific morphogenetic movements and maintains the integrity of epithelia undergoing mechanical stress (for a review, see Tepass, 1999). Previous studies showed that precise regulation of E-Cad in follicle cells is required for oocyte positioning, centripetal migration and border cell migration (Godt and Tepass, 1998; Niewiadomska et al., 1999). Furthermore, EGF receptor activity has been linked with the regulation of E-cadherin expression and invasiveness in human cancer cell lines (Alper et al., 2000; Jones et al., 1996). We therefore examined E-Cad protein by confocal microscopy of *Ras* mosaic egg chambers.

We first analyzed E-cadherin protein localization in control egg chambers; as previously reported, E-Cad was localized in the follicular epithelium in a honeycomb pattern corresponding to the zonula adherens (Niewiadomska et al., 1999). Surprisingly, in addition to this apicolaterally localized E-Cad, egg chambers undergoing dorsal appendage morphogenesis also exhibited basal localization of E-Cad in an anterior swath of dorsal midline cells and in a ring of anterior columnar follicle cells (Fig. 7A) (J. D., K. J., D. Kiehart and C. B., unpublished data).

In all Ras clones examined, the levels of apical E-Cad were not noticeably altered (data not shown). Strikingly, however, a *Ras* clone opportunely positioned over the dorsal midline displayed dramatically reduced levels of basal E-Cad (Fig. 7B-E). Furthermore, wild-type cells abutting the clone had normal basal E-Cad localization except at their interface with *Ras* mutant cells (Fig. 7E), suggesting that the basal localization of E-Cad within the midline cells depends on Ras and, perhaps, basal E-Cad in neighboring cells. We confirmed the dorsal midline position of this clone by identifying the location of the

DAPI-stained oocyte nucleus (data not shown). These data demonstrate that *Ras* is required for basal localization of E-Cad on the dorsal midline.

DISCUSSION

An emerging hypothesis for embryonic dorsoventral patterning

The mosaic analyses described here both clarify and contribute new information to the general understanding of dorsoventral patterning in *Drosophila*. In a simple model for embryonic dorsoventral patterning (for a review, see Anderson, 1998), Gurken/Egfr/Ras signaling in the egg chamber represses *pipe* transcription dorsally. *pipe* function in the ventral follicle cells is necessary to activate the serine protease cascade in the perivitelline space, which leads to activation of the Toll receptor in the embryonic plasma membrane by cleaved Spätzle (Spz) and, ultimately, a dorsoventral gradient of Dorsal nuclear translocation. Consistent with this hypothesis, loss of *pipe* function in the follicle cells leads to the absence of zygotic Twist and embryonic dorsalization, and ectopic expression of *pipe* throughout the follicular epithelium results in embryonic ventralization (Nilson and Schüpbach, 1998; Sen et al., 1998). In this work, however, we found no direct spatial relationship between dorsal *Ras* clones (and, therefore, ectopic *pipe*) in the egg chamber and ectopic Twist in the embryo.

Our data are consistent with the hypothesis that additional patterning events modify the initial asymmetry determined by Egfr signaling and thereby establish the final Dorsal gradient. Additional patterning downstream of *Egfr* has also been suggested to explain another observation, that the embryonic phenotype resulting from maternal mutations in *gurken* or *Egfr* is not simply an expansion of ventral embryonic fates, as detected by Twist expression and formation of a larger ventral furrow. Rather, many embryos exhibit a pattern duplication characterized by two ventrolateral regions of maximum nuclear Dorsal and Twist, separated by a ventral minimum, which leads to the eventual invagination of two furrows (Roth and Schüpbach, 1994; Schüpbach, 1987). Our result that *Ras* clones cell-autonomously derepress *pipe* suggests that such a refinement process occurs downstream of *pipe*.

Consistent with our findings, Morisato (Morisato, 2001) demonstrated that the additional patterning events that govern the shape of the Dorsal gradient in wild-type embryos (and can create two furrows in ventralizing mutants) do not occur in the follicular epithelium. Rather, the refinement process occurs at the level of Spätzle processing or Toll activation in the embryo. Furthermore, elegant mosaic analyses carried out by Nilson and Schüpbach (Nilson and Schüpbach, 1998) reveal that, while loss-of-function *pipe* or *windbeutel* (*wind*) clones on the ventral side of the egg chamber result in a local loss of ventral Twist expression in the embryo, ventral clones of *pipe* or *wind* also exert a non-autonomous effect on lateral embryonic fates. These investigators hypothesize that an 8-12 cell wide ventral region in the egg chamber (defined by the spatial requirement for *pipe*) generates ventral information, perhaps imbedded in the vitelline membrane, which is then competent to establish the Dorsal gradient along the entire dorsoventral axis, presumably via outward diffusion of a ventral morphogen.

In addition, other patterning mechanisms that influence the Dorsal gradient have been discovered recently. Nuclear translocation of Dorsal can be modified by maternal Sog and Dpp in a pathway parallel to Toll signaling (Araujo and Bier, 2000). Also, the serine protease cascade upstream of Toll is subject to feedback inhibition, providing an additional level of regulation (Dissing et al., 2001; Han et al., 2000).

Our work, together with the discoveries discussed above, points to a model (Fig. 8B, top) in which the initial asymmetry generated by restriction of *pipe* to the ventral-most 40% of the egg chamber leads to the cleavage of the zymogen Spz in the ventral-most 40% of the perivitelline space. The positive ventralizing activity of C-Spz is opposed by the activity of a diffusible inhibitor, possibly N-Spz (Morisato, 2001). In wild-type patterning, this inhibitor narrows the domain of ventralizing C-Spz activity, originally determined by *pipe* expression in follicle cells, from 40% of the perivitelline space

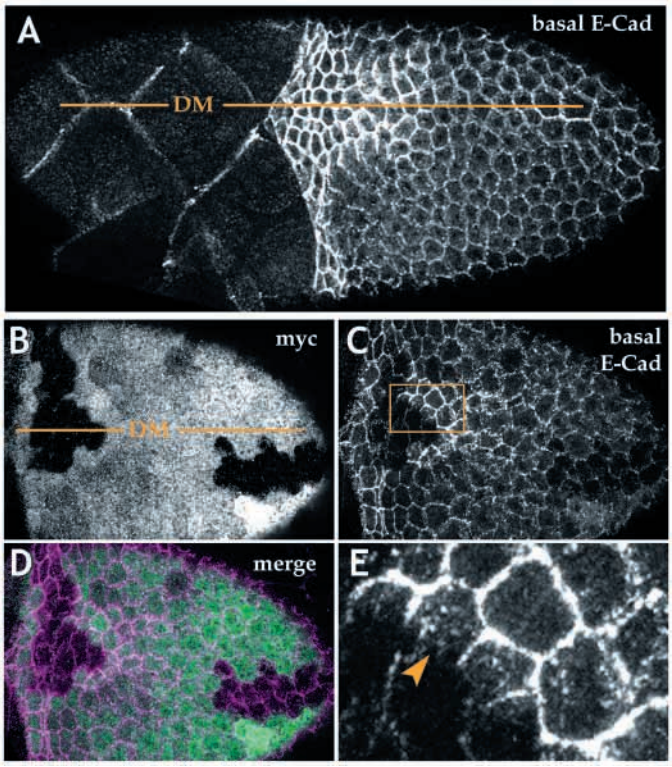

Fig. 7. Basal localization of E-Cad is disrupted in a *Ras* clone on the dorsal midline. Basal confocal projections of E-Cad fluorescence in (A) wild-type and (B-E) *Ras* mosaic egg chambers reveal a novel, *Ras*-dependent pattern of basal E-Cad localization. Anterior is to the left in all panels. DM, dorsal midline (confirmed by position of oocyte nucleus, not shown). (A) During dorsal appendage morphogenesis, E-Cad is localized basally in an anterior patch of dorsal midline follicle cells as well as several rows of anterior follicle cells encircling the egg chamber. Note that all cells exhibit some degree of basal E-Cad fluorescence, but the level of basal localization in midline and anterior cells is much higher. (B) This stage 10B mosaic egg chamber bears two *Ras* clones, marked by lack of myc. The anterior clone resides in the dorsal midline region that normally displays basal E-Cad. (C,D) Basal E-Cad is greatly reduced in the *Ras* mutant cells. (E) An enlargement of the boxed region in C reveals the cell non-autonomous effect of the *Ras* mutant clone on basal E-Cad protein in neighboring wild-type cells, which have lost basal E-Cad on cell surfaces abutting the *Ras* clone (arrowhead).

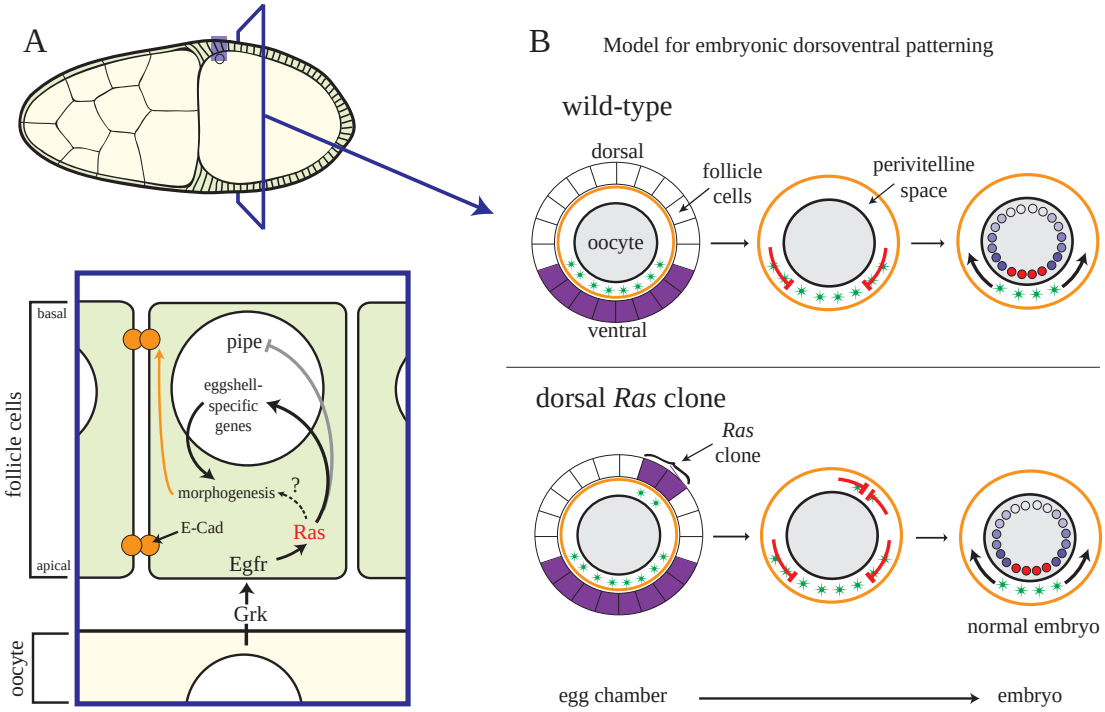

Fig. 8. (A) *Ras* is required for *pipe* repression and dorsal appendage morphogenesis. Higher levels of Ras protein are required for the dorsoventral patterning of the eggshell (black arrow) than for embryonic dorsoventral patterning, which begins with the cell-autonomous repression of *pipe* by Ras signaling (gray ⊣ line) in dorsal and lateral follicle cells. This eggshell vs. embryo difference in requirement for *Ras* may be due to an eggshell-specific requirement for higher levels of Ras during Egfr signal amplification. Ras is required for dorsal follicle cell morphogenesis either through transcriptional activation of morphogenesis genes (solid black arrow) or through the direct cytoplasmic activation of cytoskeletal or adhesion molecules (dashed black arrow). One outcome of Ras signaling is the basal localization of E-Cad in dorsal midline cells. (B) A hypothesis for embryonic dorsoventral patterning: dorsal Egfr signaling restricts *pipe* mRNA (purple) to the ventral-most 40% of follicle cells. Pipe-positive cells activate a serine protease cascade in the perivitelline space separating the vitelline membrane (orange) and the oocyte. This cascade culminates in cleavage of the Spz zymogen to produce the ventral determinant C-Spz (green asterisks), the activity of which is negated by N-Spz and/or other inhibitors (red ⊣ lines). This inhibition process normally reduces the ventral-determining region to 20% of the embryonic circumference, and, in embryos from *Ras* mosaic egg chambers, can completely overcome isolated regions of ectopic ventral activity caused by a *Ras* clone. This 'ventral-most' 20% region then instructs the nuclear Dorsal gradient along the dorsoventral axis (blue nuclei), and highest levels of nuclear Dorsal result in Twist protein expression (red nuclei).

to approximately 20%. This narrower region of ventralizing activity then activates Toll in a graded fashion, defining the final shape of the Dorsal gradient.

In *Ras* mosaic egg chambers (Fig. 8B, bottom), *pipe* is expressed ectopically in dorsal *Ras* clones, but is prevented from activating Twist because the size or width of the clone is too small to overcome the action of the inhibitor. Theoretically, this inhibitor could be present throughout the perivitelline space but overcome only by a large enough pool of C-Spz. Alternatively, as Morisato proposed (Morisato, 2001), the inhibitor could itself be a byproduct of Spz cleavage, emanating only from sources of ventralizing activity. If this mechanism is correct, then our data predict that, until the pool of C-Spz reaches a certain size, the inhibitor is more potent than C-Spz. We hypothesize that only very large dorsal *Ras* clones could exceed the size or width threshold needed to overcome the inhibitor and cause ectopic expression of Twist in the embryo. In the rare cases in which embryos ectopically expressed Twist, the region of ectopic Twist was always found in contact with the normal ventral domain of Twist. We hypothesize that these embryos result from egg chambers in which *Ras* clones overlap the edge of the normal *pipe* expression domain and alter the shape of the domain by creating a local bulge in *pipe* expression. The inhibitor downstream of *pipe* may then narrow that distorted ventral shape to produce a corresponding, though smaller, bulge in the Twist domain.

Support for this hypothesis comes from the analysis of egg chambers with ventral *wind* clones surrounding small 'islands' of wild-type cells in ventral-most positions (Nilson and Schüpbach, 1998). In these instances, if the wild-type 'island' is less than 4-6 cells wide, it is not able to induce Twist expression in the embryo. This situation is similar to our result that dorsal *Ras* clones fail to induce Twist. Together, our data suggest that ventral information from small patches of cells expressing *pipe* can be 'swamped out' by neighboring dorsally-fated cells, probably through the function of a diffusible inhibitor.

Embryonic dorsoventral patterning is robust

The ventralizing activity of ectopic *pipe* expression in *Ras* mosaic egg chambers can be overcome by post-*pipe* patterning events. The existence of this compensatory mechanism demonstrates that embryonic dorsoventral patterning is a robust process. This resilience is generated by the action of additional rounds of pattern refinement, which may help to

buffer these important early events of embryonic development from perturbations in signaling caused by genetic defects or environmental factors. Furthermore, the zygotic genes that orchestrate dorsoventral patterning (*Dpp*/*BMP4* and *Sog*/*chordin*) are conserved between arthropods and chordates, and, extraordinarily, their biological function in dorsoventral patterning is also conserved (for a review, see Ferguson, 1996). However, where flies use post-*pipe* pattern refinement to buffer the Dorsal gradient, which determines the Dpp/Sog pattern, chordates use a completely different developmental pathway to arrive at the BMP4/chordin patterning event. Consequently, the specific mechanism of dorsoventral 'buffering' in flies cannot be shared by chordates, suggesting that each group evolved unique mechanisms to buffer the conserved aspects of dorsoventral patterning. Understanding the degree of diversity among buffering systems will be useful in determining evolutionary relationships and will facilitate studies examining the evolution of developmental mechanisms.

Eggshell versus embryo

To explain the differential phenotype of eggs laid by females transheterozygous for strong hypomorphic alleles of *Ras*, we hypothesized that either eggshell patterning is more sensitive than embryonic patterning to reductions in Ras, or a Ras-independent Egfr effector pathway mediates embryonic patterning. The cell-autonomous derepression of *pipe* in *Ras* null clones demonstrates that *Ras* is required to initiate embryonic dorsoventral patterning and suggests that the first hypothesis is correct. Conversely, the result that embryos developing from *Ras* mosaic egg chambers are rarely abnormal supports the second hypothesis, with the added complexity that the Ras-independent effector pathway must also bypass *pipe*. As described above, however, our data suggest that very large Ras clones, or an entirely mutant epithelium, would induce ectopic Twist expression in the embryo and lead to severe embryonic dorsoventral patterning defects.

Indeed, in five of 26 cases, *Ras* follicle cell clones did result in ectopic Twist in the embryo, presumably because those clones were large enough to overcome the post-*pipe* patterning events that dampen the ventralizing effects of smaller clones. Furthermore, ectopic expression of *pipe* throughout the follicular epithelium is indeed sufficient to cause embryonic ventralization (Sen et al., 1998). Therefore, the lack of embryonic defects resulting from most *Ras* mutant follicle cell clones, which are too small to overcome post-*pipe* patterning processes, is misleading with respect to the question of whether *Ras* is required generally for embryonic dorsoventral patterning. Given these considerations, our results are more consistent with the first hypothesis, that is, *Ras* is required for dorsoventral patterning of the embryo, and eggshell patterning is more sensitive than embryonic patterning to reductions in Ras.

Despite the appeal of this conclusion, the formal possibility remains that an Egfr-dependent, Ras-independent dorsalizing factor feeds into the embryonic patterning pathway downstream of *pipe*. Such a hypothesis, however, requires complex rationalizations. For example, it conflicts with elegant experiments conducted by Sen and colleagues (Sen et al., 1998) in which ectopic dorsal expression of *pipe* in *pipe* mutant egg chambers causes reversal of embryonic dorsoventral polarity. If an Egfr-dependent, Ras-independent, post-*pipe* dorsalizing factor existed, one would predict that ectopic dorsal expression of *pipe* in a *pipe* mutant egg chamber would exert little to no effect on the pipe mutant phenotype. Furthermore, numerous screens for dorsoventral patterning mutants have failed to recover components of known Ras-independent Egfr effector pathways. Thus, we favor the hypothesis that *Ras* is required to establish embryonic dorsoventral polarity.

Eggshell sensitivity and Ras specificity

Why would eggshell patterning be more sensitive than embryonic patterning? Furthermore, why would that sensitivity be specific to reductions in Ras and not Gurken or Egfr? Two hypotheses can explain why eggshell patterning would be acutely sensitive specifically to reductions in Ras. First, dorsoventral patterning of the eggshell requires amplification and modulation of Egfr activity, achieved by autocrine signaling involving Spitz, Rhomboid, Vein, and Argos, to define two dorsolateral populations of follicle cells. These post-Gurken patterning events are eggshell-specific, while *pipe* repression is achieved solely by Gurken signaling (for a review, see Van Buskirk and Schüpbach, 1999). Furthermore, since our results support the hypothesis that low lateral levels of Gurken suffice to repress *pipe* (Pai et al., 2000), it is likely that *pipe* repression requires only low-level signaling. The hypomorphic *Ras* mutations primarily affect the transcription of *Ras* (Schnorr and Berg, 1996), which, theoretically, reduces the number of Ras molecules rather than the effectiveness of each molecule. Therefore, the hypomorphic mutant may produce enough Ras molecules to transduce the initial Gurken signal, repress *pipe* and pattern the embryo, but too few Ras molecules to transduce the high levels of Egfr signaling required for eggshell patterning. Thus, the number of Ras molecules may be limiting for eggshell patterning, which requires an intense surge of Ras signaling, but not limiting for *pipe* repression, which may be accomplished with only a trickle of signaling that is easily transduced by a small number of Ras molecules.

This 'amplification threshold' hypothesis predicts that, like Ras, Egfr mutations would cause a differential phenotype since Egfr is also involved in eggshell-specific signal amplification. Egfr mutations, however, affect eggshell and embryonic patterning equally. The nature of the *Egfr* and *Ras* alleles used in the analysis of dorsoventral phenotypes may provide an explanation. The *Ras* hypomorphic mutations analyzed by Schnorr and Berg (Schnorr and Berg, 1996) are lethal EMS mutations in trans to a P-element insertion that simply reduces the level of the wild-type *Ras* transcript. By contrast, viable *Egfr* alleles such as top^{1}, top^{CJ}, and top^{EE38} specifically affect the ligand-binding domain of the receptor (Clifford and Schüpbach, 1994). In addition, these alleles were isolated in mutagenesis screens that demanded viability (Schüpbach, 1987). Therefore, since the Egfr ligands Spitz and Vein are required throughout embryonic and larval development whereas Gurken is required only in oogenesis, the *Egfr* alleles isolated in such a screen are likely to disproportionately affect Gurken signaling. Since Gurken initiates both eggshell and embryonic patterning, while the other ligands bring about eggshell-specific Egfr signal amplification, these unique *Egfr* alleles would be expected to compromise eggshell and embryonic patterning equally. We speculate that a genuine hypomorphic mutation affecting the overall levels of Egfr protein would behave like the hypomorphic *P* allele of *Ras*

(strong eggshell defects, weak dorsoventral embryonic defects). Indeed, Clifford and Schüpbach (Clifford and Schüpbach, 1989) observed that some *Egfr* mutant females lay rare eggs with fused dorsal appendages that give rise to normal embryos.

A second hypothesis can also explain why eggshell patterning would be more sensitive than embryonic patterning specifically to reductions in Ras. Our data demonstrate that *Ras* mutant cells fail to initiate or accomplish dorsal follicle cell morphogenesis. These phenotypes, along with our *pipe-lacZ* results, suggest that *Ras* is required to establish dorsal follicle cell fate, a prerequisite for morphogenesis. Our data are also consistent with an additional requirement for *Ras* specifically regulating morphogenesis. Gurken and Egfr, however, may be directly required only for patterning and may therefore influence morphogenesis only indirectly. In support of this hypothesis, the Nrk receptor tyrosine kinase, identified as an *Enhancer of Ras* in eggshell development, may provide an independent Ras pathway input specific for morphogenesis (Schnorr et al., 2001). Thus, a *Ras* mutation that produces weak embryonic defects may dramatically disrupt eggshell structures by affecting both patterning and morphogenesis. This synergism may compromise dorsal appendage morphology enough to resemble the eggshell phenotype of a severe *gurken* or *Egfr* allele, which affects eggshell and embryo equally (Schnorr and Berg, 1996; Schüpbach, 1987).

Ras and cell migration: patterning versus morphogenesis

In addition to extending our understanding of embryonic dorsoventral patterning and defining the relative contribution of *Ras* signaling toward establishing eggshell and embryonic fates, our mosaic analyses have revealed the requirement for *Ras* during dorsal appendage morphogenesis. How might *Ras* regulate dorsal follicle cell migration (Fig. 8A)? By transducing signals for dorsal follicle cell fate, Ras may affect transcription of genes involved in morphogenesis. Notably, Broad-Complex (BR-C), Mirror, Bunched, and Fos/Kayak respond to Egfr signaling and likely affect transcription of genes involved in dorsal appendage formation (Deng and Bownes, 1997; Dequier et al., 2001; Dobens et al., 1997; Jordan et al., 2000). Alternatively, Ras activity may directly affect key cytoskeletal or adhesion molecules that play active roles during the morphogenetic process. Since many of the events of dorsal follicle cell morphogenesis occur quite rapidly – stage 11, for example, encompasses profound morphogenetic changes (J. D., K. J., D. Kiehart and C. B., unpublished data), but is completed in less than 30 minutes (Lin and Spradling, 1993), and since MAP kinase activity is dynamic during the early stages of morphogenesis (Peri et al., 1999), we suspect that Ras signaling directly modulates the activity of migration molecules. Most likely, *Ras* functions in dorsal follicle cell migration through some combination of direct cytoplasmic effects and transcriptional regulation.

To understand the role of *Ras* in cell migration, we can look to other migration events controlled by Ras signaling. In *Drosophila*, disruptions in Ras signaling can hinder the migration of a subset of follicle cells called border cells (Duchek and Rørth, 2001; Lee et al., 1996). Significantly, border cell fate remains properly specified in these experiments, revealing migration-specific functions for *Ras*. Importantly, the movement of dorsal follicle cells differs significantly from that of border cells, which navigate through germline cells as a small epithelial patch (Niewiadomska et al., 1999). Dorsal appendage formation involves the coordinated morphogenetic movements of an epithelial sheet (J. D., K. J., D. Kiehart and C. B., unpublished data). These differences demonstrate that each migration event offers a unique opportunity to study the role of Ras during developmentally regulated cell migration in *Drosophila*.

Ras effectors in dorsal follicle cell morphogenesis: a novel role for E-cadherin?

What are the effectors of Ras during dorsal follicle cell migration? One possibility is E-cadherin, since *Ras* is required for the basal localization of this molecule in midline follicle cells. Importantly, the levels of apicolaterally-localized E-Cad appear normal in *Ras* clones. This result suggests that Ras signaling does not regulate the canonical adherens-junction function of E-Cad, which provides integrity to the epithelium during morphogenesis. Instead, *Ras* affects the basal localization of E-Cad on the dorsal midline, which may anchor the midline cells or otherwise influence the mechanical movements of the dorsal appendage primordia.

The loss of basal E-Cad in a *Ras* clone on the midline was surprising because the dorsal midline is thought to be a region of significantly diminished Egfr activity (Peri et al., 1999). Why, then, would *Ras* be required there for the localization of an adhesion molecule? Perhaps Ras signaling is actually active on the dorsal midline between stages 10B and 12. Alternatively, a history of high and then low Egfr/Ras signaling may be required for basal E-Cad protein localization in midline cells. Further exploration of the precise regulation of E-Cad during dorsal follicle cell morphogenesis is needed to elucidate the relationship between Ras signaling and E-Cad localization, and to address whether basal localization of E-Cad in anterior and midline cells is required for the proper morphogenesis of dorsal appendages.

In addition to E-Cad, *Ras* may regulate other adhesion, signaling, or cytoskeletal molecules to permit or instruct dorsal follicle cell migration. Known cellular effectors of Ras in mammalian cells include c-Raf, RalGDS, and PI 3 kinase (for a review, see Bar-Sagi and Hall, 2000). To identify molecules that interact with *Ras* in *Drosophila* follicle cells, Schnorr and colleagues (Schnorr et al., 2001) screened for dominant enhancers of a weak *Ras* eggshell phenotype. They identified 13 strong enhancers that can be grouped into two major molecular categories: signaling and cytoskeleton. Interestingly, two enhancers, *dock* and *Tec29A*, encode signaling molecules that directly regulate cytoskeletal function (Garrity et al., 1996; Guarnieri et al., 1998; Rao and Zipursky, 1998; Roulier et al., 1998). Studies that separate cell fate specification from morphogenetic events are needed to determine whether *Ras* actively controls the morphogenesis of dorsal follicle cells.

In conclusion, our finding that *Ras* is required for *pipe* repression argues against the hypothesis that a Ras-independent pathway transduces Egfr signals to pattern the embryo. This result contributes to the growing body of evidence that, in *Drosophila*, Egfr signaling is transmitted to the nucleus primarily by the Ras pathway rather than by alternative effector molecules. Furthermore, our data

demonstrate that dorsoventral patterning is buffered by post-*pipe* patterning events that define the final shape of the embryonic dorsoventral gradient. Additionally, we show that Ras is required for dorsal appendage patterning and morphogenesis as well as for the proper subcellular localization of E-cadherin, a major epithelial adhesion protein. Ras signaling is linked to cell migration in many developmental and disease contexts, providing justification for further dissection of Ras pathway function during dorsal appendage morphogenesis in *Drosophila*.

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