Development 128, 4553-4562 (2001)
Printed in Great Britain © The Company of Biologists Limited 2001
DEV5915

Establishment of dorsal-ventral polarity of the *Drosophila* egg requires *capicua* action in ovarian follicle cells

Deborah J. Goff[1], Laura A. Nilson[2] and Donald Morisato[1,*]

[1]Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, 240 Longwood Avenue, Boston, MA 02115, USA
[2]Department of Biology, McGill University, 1205 Avenue Docteur Penfield, Montreal QC H3A 1B1, Canada
*Author for correspondence (e-mail: dmorisato@hms.harvard.edu)

Accepted 1 August 2001

SUMMARY

The dorsal-ventral pattern of the *Drosophila* egg is established during oogenesis. Epidermal growth factor receptor (Egfr) signaling within the follicular epithelium is spatially regulated by the dorsally restricted distribution of its presumptive ligand, Gurken. As a consequence, *pipe* is transcribed in a broad ventral domain to initiate the *Toll* signaling pathway in the embryo, resulting in a gradient of Dorsal nuclear translocation. We show that expression of *pipe* RNA requires the action of *fettucine* (*fet*) in ovarian follicle cells. Loss of maternal *fet* activity produces a dorsalized eggshell and embryo. Although similar mutant phenotypes are observed with regulators of Egfr signaling, genetic analysis suggests that *fet* acts downstream of this event. The *fet* mutant phenotype is rescued by a transgene of *capicua* (*cic*), which encodes an HMG-box transcription factor. We show that Cic protein is initially expressed uniformly in ovarian follicle cell nuclei, and is subsequently downregulated on the dorsal side. Earlier studies described a requirement for *cic* in repressing zygotic target genes of both the *torso* and *Toll* pathways in the embryo. Our experiments reveal that *cic* controls dorsal-ventral patterning by regulating *pipe* expression in ovarian follicle cells, before its previously described role in interpreting the Dorsal gradient.

Key words: Oogenesis, Embryogenesis, HMG box, *Egfr*, *pipe*, *Drosophila*

INTRODUCTION

Dorsal-ventral (DV) patterning of the *Drosophila* embryo begins during oogenesis and relies on a series of intercellular signaling events in the egg chamber between the germline-derived oocyte and the somatically derived follicle cells (Ray and Schüpbach, 1996; Nilson and Schüpbach, 1999). The initial asymmetry for embryonic DV polarity is established at mid-oogenesis in the oocyte and is subsequently conveyed to the surrounding follicle cells. Spatial information generated within the follicular epithelium is then transmitted back to the germline, and is interpreted by the embryo after fertilization.

The initial polarizing signal from the oocyte to the follicle cells is determined by *gurken* (*grk*), which encodes a secreted transforming growth factor α (TGFα)-like protein (Neuman-Silberberg and Schüpbach, 1993). During mid-oogenesis, *grk* mRNA associated with the oocyte nucleus is localized to the dorsal anterior corner of the oocyte, resulting in restricted distribution of Grk protein and the activation of the Epidermal growth factor receptor (Egfr) in dorsal follicle cells.

Activated Egfr transduces the dorsal signal to follicle cell nuclei via the Ras-Raf-mitogen-activated protein (MAP) kinase kinase - MAP kinase cascade (Brand and Perrimon, 1994; Hsu and Perrimon, 1994; Schnorr and Berg, 1996; Wasserman and Freeman, 1998; Peri et al., 1999). The pattern of Grk-mediated Egfr activation can be visualized by target gene RNA expression in a dorsal-anterior patch of follicle cells centered on the dorsal midline (Ruohola-Baker et al., 1993; Musacchio and Perrimon, 1996; Sapir et al., 1998). Egfr activation in the follicle cells produces two delayed consequences: generating DV polarity of the embryo and patterning the eggshell dorsal appendages (Roth, 1998).

The DV pattern of the embryo is ultimately determined by the domain of *pipe* expression in the follicle cells during oogenesis (Nilson and Schüpbach, 1998; Sen et al., 1998). Expression of *pipe* RNA is normally restricted to ventral follicle cells, as a result of repression in dorsal follicle cells by Egfr activation (Sen et al., 1998). Pipe is expressed in the Golgi (Sen et al., 2000) and is predicted to act as a glycosaminoglycan-modifying enzyme on an undetermined substrate. This modified molecule is hypothesized to ventrally activate an extracellular serine protease cascade, resulting in the proteolytic processing of Spätzle protein, which is thought to act as a ligand for the embryo transmembrane receptor Toll (Morisato and Anderson, 1995; LeMosy et al., 1999). Toll signaling is relayed by cytoplasmic components to effect nuclear translocation of the Dorsal protein, a member of the NF-κB/*rel* family (Roth et al., 1989; Rushlow et al., 1989; Steward, 1989). The maternal DV pathway culminates in a ventral-to-dorsal gradient of nuclear Dorsal, resulting in the

transcriptional activation or repression of zygotic genes that respond to distinct nuclear concentrations of Dorsal and thereby subdividing the DV axis into discrete stripes (Rusch and Levine, 1996).

Mutations in *grk* or *Egfr* cause an expansion of ventral structures at the expense of dorsal pattern elements in both the eggshell and embryo (Schüpbach, 1987). Conversely, mutations in genes encoding regulators of the Egfr pathway produce a dorsalized eggshell and embryo. This phenotype is apparently caused by ectopic Egfr signaling, as constitutive activation of the Egfr by artificial dimerization also produces a dorsalized eggshell and embryo (Queenan et al., 1997). The dorsalizing mutations can be divided into two groups. The first class of genes, which includes *K10* and *sqd* (Wieschaus et al., 1978; Kelley, 1993), is required in the germline for *grk* mRNA association with the oocyte nucleus. Loss of function in these genes results in *grk* mRNA mislocalization around the entire anterior cortex of the oocyte (Neuman-Silberberg and Schüpbach, 1993), and ectopic Egfr activation in the follicle cells (Ruohola-Baker et al., 1993; Kelley, 1993). The second class of genes, which includes *kek1* and *Cbl*, acts in the follicle cells to negatively regulate the *Egfr* pathway (Ghiglione et al., 1999; Pai et al., 2000). In these mutants, Egfr signaling fails to be downregulated, resulting in ectopic activation of *Egfr* pathway target genes.

The *grk-Egfr* signaling pathway coordinately establishes the polarity of both the eggshell and embryo. Yet, the analysis of genes that act downstream of Egfr signaling in the follicle cells indicates that the pathway bifurcates into one branch which regulates eggshell pattern and one branch which establishes embryo polarity. For example, while mutations in *argos* change spacing of the dorsal appendages without affecting the pattern of the embryo (Wasserman and Freeman, 1998), mutations in *pipe* lead to dorsalized embryos without affecting the eggshell pattern (Stein et al., 1991).

We describe new maternal effect mutations in a locus named *fettucine* (*fet*) that we show are alleles of the *capicua* (*cic*) gene. Mutant *fet* females lay dorsalized eggs due to a requirement for *fet* in ovarian follicle cells. Unlike other dorsalizing mutations, *fet* does not act as a negative regulator of the *Egfr* pathway in patterning the embryo. Rather, *fet* appears to have distinct functions in each pathway downstream of the branchpoint for embryo and eggshell patterning. In embryo patterning, *fet* is required for *pipe* RNA expression in ventral follicle cells. In eggshell patterning, *fet* is required for modulating Egfr signaling later during oogenesis. *cic* has previously been shown to encode an HMG-box transcription factor (Jiménez et al., 2000). In support of the genetic analysis described above, we show that Cic protein is expressed in ovarian follicle cell nuclei. In addition to the maternal effect phenotypes, mutant *fet* flies exhibit zygotic wing defects, suggesting that *cic* is required in several developmental processes.

MATERIALS AND METHODS

Genetics

We isolated P element alleles of *fet* in a hybrid dysgenic screen for suppressors of spz^{D1} sterility (Morisato and Anderson, 1994). P-type π2 spz^{D1}/ *TM6* males were crossed to M-type *DTS/ TM3, Sb* females. Male progeny of the genotype * spz^{D1}/ *TM3, Sb* were crossed to *TM1/ TM3, Ser* females to generate * spz^{D1}/ *TM1* and * spz^{D1}/ *TM3, Ser* females. From 37,000 females screened for fertility, 16 lines were recovered: five lines mapped to *spz* and 11 lines mapped to chromosome III, but were unlinked to *spz*. Five of the 11 second-site dominant suppressors, including fet^{E11}, were alleles of *fet.*

We isolated EMS alleles of *fet* in an F_1 screen for wing defects. Females of the genotype fet^{E11}/ *TM3, Sb* were crossed to mutagenized *st ry e* males, and 20,000 *st ry e* */ fet^{E11} flies were screened for extra wing veins or blisters. Five mutant lines were recovered, including fet^{T6} and fet^{U6}, and all exhibited recessive maternal effect phenotypes affecting embryonic pattern. The fet^{U6} allele behaves genetically like a null allele, as the maternal effect and zygotic wing defects observed with fet^{E11} in *trans* to fet^{U6} flies were indistinguishable from fet^{E11} in *trans* to *Df(3R) Dl*^{BX12}, a deficiency that uncovers the *fet* locus.

For mosaic analysis, *fet* alleles were recombined onto a chromosome containing *FRT82B* (Xu and Rubin, 1993). For making germline clones, *FRT82B fet/ TM3, Sb* females were crossed to *hs-FLP/ Y; FRT82B* ovo^{D1}/ *TM3, Sb* males (Chou et al., 1993), and the progeny were heat-shocked as third instar larvae for 2 hours at 37°C on 3 consecutive days. Eggs were collected from non-stubble female progeny crossed to wild-type males. For making unmarked follicle cell clones, *e22c-GAL4, UAS-FLP/ CyO; FRT82B* flies (Duffy et al., 1998) were crossed to *FRT82B fet/ TM3, Sb* flies. Eggs were collected from non-curly, non-stubble female progeny crossed to wild-type males. Even under optimal conditions, only a fraction of the egg chambers were expected to contain follicle cell clones. For making marked follicle cell clones, eggs laid by mosaic females of the genotype dec^{VA28} *hs-FLP/* dec^{VA28}*; FRT82B* fet^{U6}*/FRT82B P[*dec^{+}*]* were inspected for the presence of *dec*-marked clones and associated embryonic DV patterning defects, recognized by reduction or absence of ventral cuticular markers (Nilson and Schüpbach, 1998).

For rescue of the *fet* phenotype, we used a P-element transgene encoding the *cic* transcription unit, which was inserted on chromosome II (kindly provided by Gerardo Jiménez and Jordi Casanova, Institut de Biologia Molecular de Barcelona).

Analysis of ovaries and embryos

Enhancer trap lines were used for detecting the expression of *kek1* (*15A6*) (Musacchio and Perrimon, 1996) and *mirror* ($mirr^{6D1}$) (Ruohola-Baker et al., 1993). Briefly, ovaries were dissected from aged females, separated into ovarioles and fixed in formaldehyde/ heptane for 10 minutes. The expression of β-galactosidase was detected by incubating the ovaries in 0.2% X-Gal staining solution overnight at 37°C. Stained ovaries were dissected into individual egg chambers and mounted in 75% glycerol.

For analysis of *grk* RNA expression in ovaries, a digoxigenin-labeled DNA probe was prepared using Boehringer Mannheim reagents from a cDNA template, kindly provided by Trudi Schüpbach (Princeton University). Ovaries were fixed, treated with 50 μg/ml proteinase K for 4 minutes, and hybridized after a modification of the embryo hybridization protocol (Neuman-Silberberg and Schüpbach, 1993; Tautz and Pfeifle, 1989). For detection of *fringe* and *pipe* RNA expression in follicle cells, antisense RNA probes were prepared from cDNAs, kindly provided by Ken Irvine (Waksman Institute; *fringe*) and David Stein (University of Texas, Austin; *pipe*). Ovaries were dissected into individual egg chambers, treated with 50 μg/ml proteinase K for 3 minutes, and hybridized according to Suter and Steward (Suter and Steward, 1991) with modifications (Hong and Hashimoto, 1995).

Analysis of Cic expression in ovaries was carried out with rat anti-Cic antibodies generated against the C-terminal half of Cic protein, kindly provided by Gerardo Jiménez and Jordi Casanova. Preabsorbed antiserum was used at a dilution of 1:900 with ovaries that were fixed and pretreated as described (Peri et al., 1999). Primary antibodies were visualized with biotin-conjugated anti-rat antibodies and streptavidin-horseradish peroxidase (HRP) using Vectastain ABC (Vector Laboratories). DAB staining was enhanced with NiCl as

described (Patel, 1994). For analysis of cross-sections, egg chambers were embedded in Spurr (Polysciences) and 10 μm sections containing the oocyte nucleus were identified.

For analysis of RNA expression in embryos, RNA probes were generated from *zen*, *sog* and *tll* cDNAs, kindly provided by Mike Levine (UC, Berkeley; *zen*), Ethan Bier (UC, San Diego; *sog*) and Jordi Casanova (*tll*), and in situ hybridization carried out following the protocol of Tautz and Pfeifle (Tautz and Pfeifle, 1989).

For analysis of Twist expression, embryos were stained with rabbit anti-Twist antibodies kindly provided by Siegfried Roth (Universität zu Köln). Primary antibodies were visualized with biotin-conjugated anti-rabbit antibodies and streptavidin-HRP using Vectastain ABC.

Molecular biology

Ovarian RNA was isolated from dissected ovaries by homogenizing in 10% Sarcosyl/0.5 mg/ml proteinase K and extracting in 50:49:1 phenol:chloroform:isoamyl alcohol. Poly(A)$^+$ RNA was selected by oligo (dT)-cellulose chromatography. The RNA blot was probed with a 3.5 kb *Not*I-*Eco*RI *cic* cDNA fragment containing the HMG box, kindly provided by Celeste Berg (University of Washington).

For sequencing the *fet* alleles, six overlapping PCR products (approximately 1.3 kb each) spanning the *cic* gene were amplified from genomic DNA, leaving gaps of approximately 50 bp uncovered in the second intron and 720 bp uncovered in the large fifth intron. Primers were chosen by consulting the *cic* cDNA sequence (Jiménez et al., 2000) and the *cic* genomic sequence CG5067 from the annotated genome sequence (Adams et al., 2000). Genomic DNAs for use as templates in the PCR were isolated (Jowett, 1986) from three sources: the parental stock *st ry e*, homozygous fet^{T6} pupae and heterozygous fet^{U6}/ *st ry e* adults. All six PCR products from each genotype were sequenced directly by automated PCR sequencing reactions using an ABI 373S DNA sequencer in the BCMP Biopolymers Laboratory.

In comparing the *st ry e* sequence with the published sequences, nine polymorphisms within the coding region were detected: five silent changes, three changes producing amino acid changes, and an insertion of nine nucleotides coding for an additional three glutamines in a run of seven glutamines in the N terminus. Only a single change was found between the parental chromosome and the *T6* or *U6* chromosome, as described in the Results. In the case of *T6*, there is a 5 bp repeat around the splice donor and acceptor sites of *cic* intron 3, making the junction sequence difficult to predict. Unlike all other *cic* introns, the intron 3 junction does not conform to the GT-AG rule that specifies the 5′ and 3′ ends of the intron. The closest fit to this rule positions the *T6* mutation as the last nucleotide of exon 3.

RESULTS

fettucine mutations cause a dorsalized maternal effect phenotype

We originally isolated a class of dominant suppressors of a weakly ventralizing mutation (spz^{D1}) in a dysgenic screen (Materials and Methods). These suppressors mapped to 92D and defined a new locus that we called *fettucine* (*fet*). The fet^{E11} mutation is a representative allele caused by the insertion of a P element. In a subsequent screen, the EMS-induced alleles fet^{T6} and fet^{U6} were generated. While embryos laid by spz^{D1} / + females were ventralized and failed to hatch, about 10% of eggs laid by *fet* spz^{D1}/++ females hatched. Flies carrying the fet^{E11} allele were viable as homozygotes and as transheterozygotes with fet^{U6} and fet^{T6}, and these females exhibited a recessive maternal effect phenotype in which the eggshell and embryo were dorsalized. The fet^{U6} allele behaved genetically like a null allele and was larval lethal, while the

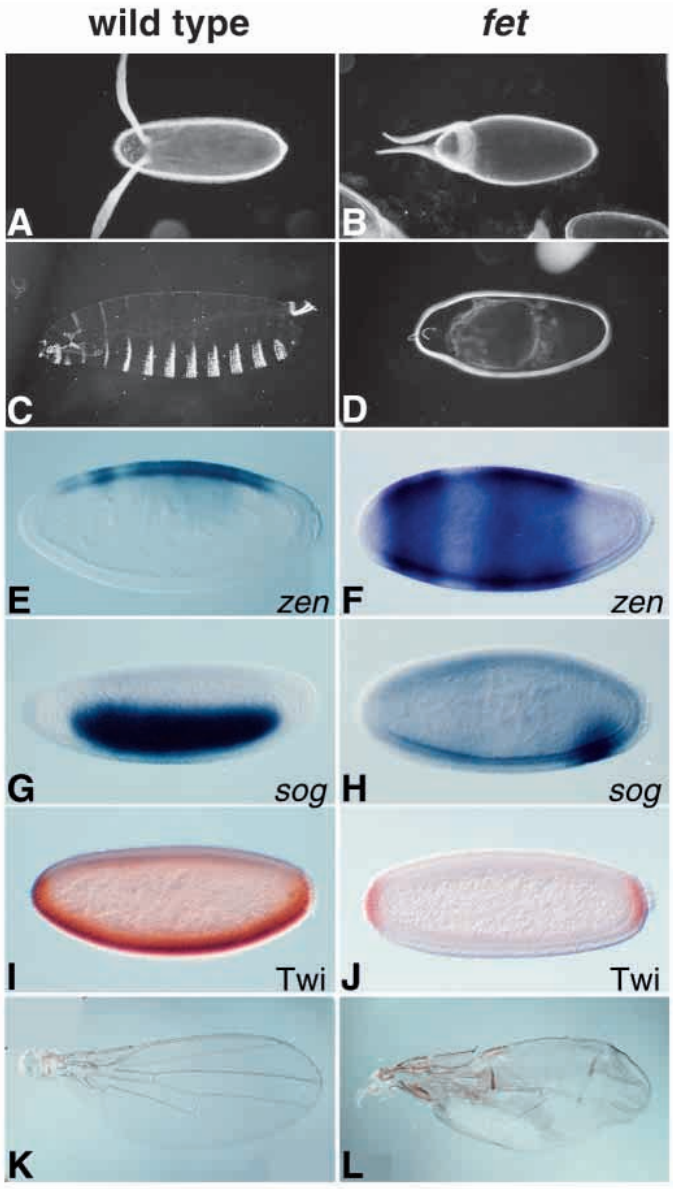

Fig. 1. Reduction of *fet* function produces a dorsalized maternal effect phenotype and zygotic wing defect. (A,B) Eggshell produced by wild-type and fet^{U6}/fet^{E11} female (dorsal view). The *fet* egg is shorter than wild type, with broader dorsal appendages that are shifted laterally and appendage base material encircling the anterior circumference. In all panels depicting an eggshell or embryo, anterior is towards the left. (C,D) Cuticle of embryo produced by wild-type and fet^{U6}/fet^{E11} female. The *fet* embryo is dorsalized, and lacks ventral and lateral denticle bands. (E,F) Expression of *zen* RNA in blastoderm embryo produced by wild-type and fet^{T6}/fet^{E11} female (lateral view, dorsal side upwards). The domain of this dorsal marker is expanded along the entire DV axis in the *fet* embryo. (G,H) Expression of *sog* RNA in blastoderm embryo produced by wild-type and fet^{T6}/fet^{E11} female. In this lateral view, only one of the two stripes expressed in the wild-type embryo is visible. No *sog* RNA is detected in the *fet* embryo except for a posterior spot. (I,J) Expression of Twist protein in blastoderm embryo produced by wild-type and fet^{T6}/fet^{E11} female. This ventral marker is not detected in the *fet* embryo, except for expression at the termini, which is regulated independently of the DV pathway. (K,L) Wing blade of wild-type and fet^{U6}/fet^{E11} fly. The *fet* wing is smaller than wild-type, and exhibits vein thickening and blistering.

fet^{T6} allele was a strong hypomorph, and homozygotes died as pharate adults.

The *fet* eggshell morphology was dorsalized, as assessed by a lateral shift of broadened dorsal appendages. In the strongest mutant combinations, ectopic dorsal appendage base material was secreted around the anterior circumference of the egg (Fig. 1A,B). Embryos produced by *fet* females (we will hereafter refer to these embryos as *fet* mutant embryos) exhibited an expansion of dorsal cell fates around the circumference of the embryo. These *fet* embryos failed to hatch and secreted a cuticle which consisted entirely of dorsal epidermis lacking any structures derived from lateral or ventral regions (Fig. 1C,D). This cuticular phenotype was preceded at the cellular blastoderm stage by expanded expression of the dorsal marker *zen* around the circumference of the embryo at the expense of the expression of the ventrolateral and ventral markers *sog* and Twist (Fig. 1E-J). Interestingly, the morphology of dorsalized *fet* cuticles was distinct from the dorsalized phenotype of *pipe* mutant cuticles (Anderson and Nüsslein-Volhard, 1986), in that the *fet* cuticles appeared broader with fewer constrictions. This observation might be accounted for by the superimposition of a segmentation defect on the dorsalized phenotype in *fet* mutants (see below).

Flies that lacked zygotic *fet* showed a spectrum of wing defects. Flies that carried weaker *fet* alleles showed ectopic vein material and blisters, while greatly thickened veins and crumpled wing blades were observed with stronger *fet* alleles (Fig. 1K,L). The severity of the wing defects correlated with the strength of dorsalization of the maternal effect phenotype.

Table 1. Egg phenotypes produced by mosaic *fet* females

Maternal genotype		Egg phenotype	
Germline	Follicle cells	Eggshell	Embryo
fet^{U6}*	Wild type	Dorsalized = 22% Wild type = 78% (*n*=1546)	Nonhatchers = 100% No development‡
fet^{E11}*	Wild type	Dorsalized = 0% Wild type = 100% (*n*=150)	Nonhatchers = 100% DV polarity normal (terminal defects)
Wild type§	*fet*^{U6}	Dorsalized = 34% Wild type = 66% (*n*=537)	Nonhatchers¶ = 54% Hatchers = 46%
Wild type§	*fet*^{E11}	Dorsalized = 0% Wild type = 100% (*n*=1082)	Nonhatchers¶ = 13% Hatchers = 87%

*Eggs were produced by females of the genotype *hs-FLP; FRT82B fet/ FRT82B ovo*^{D1}. Each egg chamber is composed of a *fet* germline and wild-type follicle cells, although *fet* follicle cell clones may have been induced as a result of *hs-FLP* action, which could account for the dorsalized eggshells observed.

‡No cellularization was observed, possibly because of problems with fertilization or early lethality.

§Eggs were produced by females of the genotype *e22c-GAL4 UAS-FLP/ +; FRT82B fet/ FRT82B*. Each egg chamber is composed of a wild-type germline and *fet* follicle cell clones, although mutant clones were not induced in every chamber (Duffy et al., 1998).

¶Of the unhatched embryos, about 10% were locally dorsalized along the anterior-posterior axis, and about 1% showed complete dorsalization.

fettucine is required in ventral follicle cells for normal DV pattern of the embryo

The observation that *fet* mutations affected both eggshell and embryo pattern suggested the involvement of *fet* in the *grk-Egfr* signaling pathway. To determine whether *fet* acts in the oocyte or the follicle cells of the egg chamber, we used FLP-FRT recombination systems to generate genetically mosaic egg chambers (Materials and Methods; Table 1), in which mutant clones were restricted to either the germline (Chou et al., 1993) or follicle cells (Duffy et al., 1998). Females with a *fet*$^{-}$ germline produced embryos showing a DV pattern that appeared normal by the expression of Twist, *zen* and *sog* (Fig. 2A,B; data not shown). However, these embryos failed to hatch, indicating an essential role for *fet* in the germline. The cuticles of these embryos (Fig. 2C) resembled a gain-of-function *torso* phenotype in which the embryonic termini were expanded at the expense of segmentation (Duffy and Perrimon, 1994) (see Fig. 6).

By contrast, mosaic females with a wild-type germline but *fet*$^{-}$ follicle cell clones produced dorsalized embryos. A few

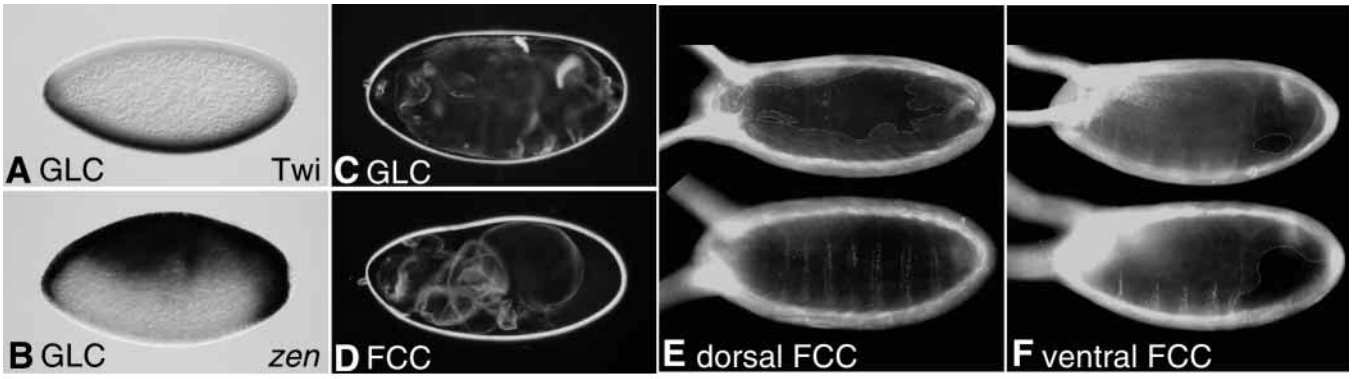

Fig. 2. *fet* is required in ventral follicle cells for normal DV pattern of the embryo. (A-C) Embryos produced by *fet* germline clones (GLC). The expression of Twist (A) and *zen* RNA (B) appear normal in *fet*^{E11} GLC embryos (lateral view, dorsal side upwards). The differentiated cuticle has normal DV polarity but shows a *torso* gain-of-function phenotype (C). (D) Embryo produced by unmarked *fet*^{U6} follicle cell clones (FCC). This cuticle of a FCC embryo is strongly dorsalized. (E,F) Embryos produced by *dec*-marked *fet*^{U6} FCC. (E) Two views of a single egg with a large dorsal clone. The dorsal follicle cells were mutant for *fet*, whereas the ventral follicle cells were wild type. The embryo within has a normal DV pattern. (F) A ventral posterior clone causes localized dorsalization, as evident by the loss of ventral denticle bands. The clone borders have been highlighted using Adobe Photoshop.

embryos were completely dorsalized (Fig. 2D), displaying a phenotype identical to embryos laid by *pipe*$^{-}$ females, while many more showed regionally dorsalized phenotypes (data not shown). In order to determine if *fet* were required in a specific region of the follicular epithelium, we generated *fet*$^{-}$ clones that were marked with a *defective chorion 1* (*dec-1*) mutation, which allowed visualization of mitotic clones within the mature eggshell (Nilson and Schüpbach, 1998). None of the dorsal or lateral clones (0/8) showed defects in the embryonic DV pattern (Fig. 2E). By contrast, ventral clones (22/24) produced local dorsalization in the embryo (Fig. 2F), implying that *fet* is required cell autonomously to establish DV polarity of the embryo. Thus, the localized requirement for *fet* resembles the behavior of *pipe* and *windbeutel*, which were previously shown to be required on the ventral side of the follicular epithelium (Nilson and Schüpbach, 1998).

The eggshell phenotypes resulting from these mosaic studies were more difficult to interpret (Table 1). Dorsalized eggshells were produced by females in which follicle cell clones had been induced, and in eggshells containing *dec*-marked clones, ectopic dorsal appendage material was observed at the anterior boundary of some *fet* clones (data not shown). Dorsalized eggshells were also produced by females carrying a mutant germline, although this observation could be attributed to the simultaneous induction of follicle cell clones as a result of *hs-FLP* action.

Taken together, these findings indicate that *fet* is required in the ventral follicle cells for normal DV pattern of the embryo. These results also suggest a requirement for *fet* in the follicle cells for patterning the eggshell, although we cannot formally rule out a possible germline contribution.

Egfr signaling appears normal in stage 10 *fet* ovaries

We further investigated the role of *fet* in *grk-Egfr* signaling by examining the spatial distribution of *grk* mRNA in the oocyte. In *fet* mutant ovaries, *grk* mRNA was properly localized to the dorsal anterior corner of the oocyte (Fig. 3A,B), an observation consistent with the normal DV pattern of *fet* germline clone embryos.

To determine the effect of *fet* mutations on *Egfr* signaling in follicle cells, we analyzed expression patterns of known *Egfr* target genes. Mutations in the negative regulator *Cbl* result in ectopic activation of the *Egfr* pathway, leading to expanded expression of *Egfr* target genes and the production of a dorsalized eggshell and embryo (Pai et al., 2000). Although mutant *fet* eggs showed a similar morphological phenotype, Egfr signaling appeared normal in the follicle cells of *fet* ovaries, as determined by the stage 10 expression pattern of *Egfr* target genes *kek1* (Fig. 3C,D) and *PZ5650* (data not shown). This result suggests that *fet* action is mechanistically distinct from that of *Cbl*, and that *fet* is not normally required for negatively regulating the *Egfr* pathway.

We also examined *fet* mutant ovaries for expression of the homeobox gene *mirror*, which is expressed in dorsal follicle cells (Jordan et al., 2000; Zhao et al., 2000). *mirror* represses *fringe* transcription, establishing a complementary domain of ventral follicle cells that expresses *fringe* RNA. Like other targets of Egfr activation, *mirror-lacZ* was expressed normally at stage 10 in *fet* ovaries (Fig. 3E,F), and *fringe* RNA expression in ventral follicle cells at this stage appeared normal

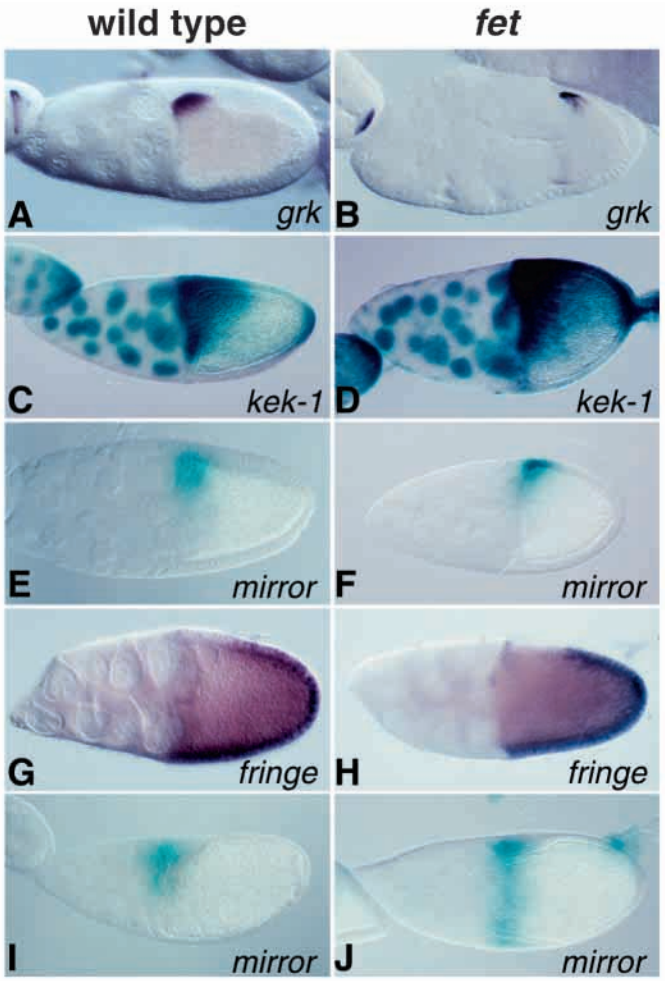

Fig. 3. Egfr signaling appears normal in *fet* ovaries at stage 10. Early stage 10 (A-H) and late stage 10 (I,J) egg chambers from wild-type (A,C,E,G,I) and fet^{U6}/fet^{E11} (B,D,F,H,J) ovaries. In early stage 10 *fet* ovaries, the expression of *grk* RNA (A,B), the *kek1* enhancer trap line *15A6* (C,D), the *mirror* enhancer trap line $mirr^{6D1}$ (E,F) and *fringe* RNA (G,H) appear normal. During late stage 10, *mirror* expression expands in *fet* ovaries (I,J). (Lateral view, dorsal side upwards.)

(Fig. 3G,H). Interestingly, however, this dorsal restriction of *mirror-lacZ* was not maintained at slightly later stages of oogenesis, when misexpression was observed around the anterior circumference of the egg (Fig. 3I,J). Taken together, these results suggest that *fet* is not required for initial reception of the Grk signal by Egfr in follicle cells.

fet acts downstream of *Egfr* for embryo patterning

We constructed double mutants of *fet* and *Egfr* to test the genetic epistasis relationship between these two genes. Females homozygous for the $Egfr^{1}$ mutation laid eggs with a ventralized eggshell and embryo (Schüpbach, 1987) (Fig. 4A,B), while *fet* females laid eggs with a dorsalized eggshell and embryo (Fig. 4C,D). Embryos laid by $Egfr^{1}$*; fet* double mutant females were dorsalized (Fig. 4E), indicating that *Egfr* requires *fet* activity to specify ventral embryonic fates and that *fet* acts downstream of *Egfr* for this function. By contrast, *fet* appears to act additively with *Egfr* for patterning the eggshell,

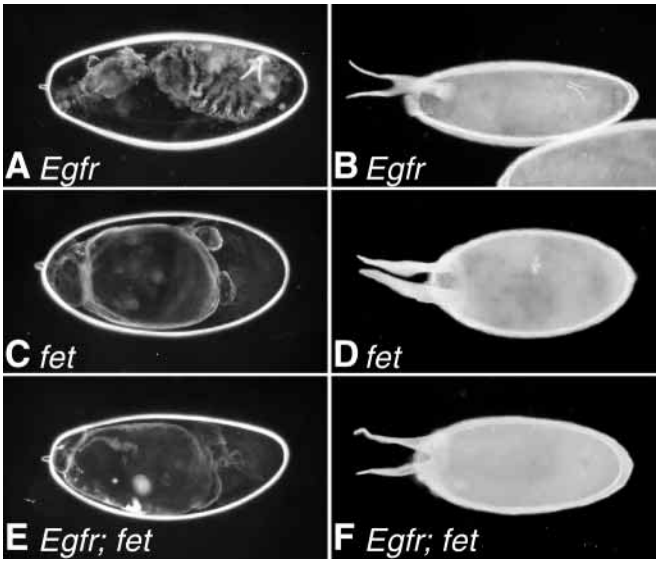

Fig. 4. *fet* acts downstream of *Egfr* in patterning the embryo. (A,B) $Egfr^{1}/Egfr^{1}$; fet^{T6}/+ females lay eggs with a ventralized embryo (A) and ventralized eggshell (B). (C,D) $Egfr^{1}$/+; fet^{T6}/fet^{E11} females lay eggs with a dorsalized embryo (C) and dorsalized eggshell (D). (E,F) $Egfr^{1}/Egfr^{1}$; fet^{T6}/fet^{E11} double mutant females lay eggs with a dorsalized embryo (E) and an eggshell (F) that shows a combination of *Egfr* and *fet* phenotypes.

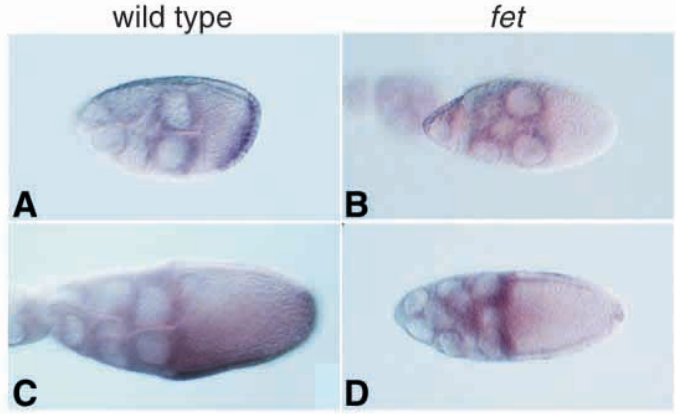

Fig. 5. *fet* is required for *pipe* expression in ventral follicle cells. Stage 9 and stage 10B egg chambers from wild-type (A,C) and fet^{U6}/fet^{E11} (B,D) ovaries were detected for *pipe* RNA expression. The oocyte nucleus is visible in the dorsal anterior corner.

as the eggshell dorsal appendages of the double mutant were broadened like *fet* dorsal appendages, but were positioned closer to the dorsal midline like *Egfr* mutant appendages (Fig. 4F).

fet is required for *pipe* expression in ventral follicle cells

The genetic observation that *fet* acts downstream of *Egfr* for defining embryo polarity led us to ask whether *fet* acts upstream or downstream of *pipe* transcription. Egfr activation establishes embryonic DV polarity by restricting *pipe* transcription to ventral follicle cells. In the wild-type ovary, expression of *pipe* RNA is first detected at stage 9 (Fig. 5A) and continues through stage 10B (Fig. 5C). In *fet* mutant ovaries, *pipe* RNA was never detected in ventral follicle cells, although expression of *pipe* RNA was observed in a few posterior cells at later stages (Fig. 5B,D). These results indicate that *fet* is required to establish *pipe* expression in the ventral follicle cells for patterning the DV axis of the embryo.

fet encodes the *capicua* HMG-box transcription factor

We mapped the *fet* mutations to the interval 67.2-68.6 on chromosome III by meiotic recombinational mapping and to 92D by P element hybridization to polytene chromosomes. As both *cic* and *bullwinkle* (*bwk*) mutations also map in this region (Jiménez et al., 2000; Rittenhouse and Berg, 1995), we examined the relationship between *fet* mutations and *cic* and *bwk* mutations.

The cic^{1} allele has a maternal effect terminal class phenotype similar to the *torso* gain-of-function phenotype, in which *tll* expression is expanded from the termini towards the center of the embryo, resulting in suppression of segmentation (Jiménez et al., 2000). *fet* mutant embryos and *fet* germline clone embryos also showed an expansion of *tll* expression (Fig. 6A-C), revealing a terminal class phenotype for *fet* embryos. This result accounted for the segmentation defect observed in the cuticles of *fet* germline clone embryos, and suggested that the *fet* mutations and cic^{1} could be allelic.

In addition to its requirement for normal terminal development, analysis of the cic^{1} allele revealed a role for *cic* in the germline as a co-factor necessary to convert Dorsal from a transcriptional activator to a repressor (Jiménez et al., 2000). In cic^{1} mutant embryos, *zen* RNA expression is expanded from its normal dorsally restricted domain to more lateral positions, although Twist expression appears wild type (Jiménez et al., 2000). By comparison, *zen* RNA expression was only slightly broadened in embryos produced by females with a *fet* germline (fet^{E11} germline clone embryos exhibited a 15% expansion of *zen* expression compared with wild type; Fig. 2B and data not shown).

We determined complementation between cic^{1} and *fet* alleles. Transheterozygous cic^{1}/fet females produced embryos with a *torso* gain-of-function phenotype (Fig. 6D), indicating that both alleles affect the same germline function. However, cic^{1} complemented the *fet* follicle cell defect, as eggs laid by transheterozygotes showed apparently normal DV polarity of both the eggshell and embryo. cic^{1}/fet flies also developed wild-type wings.

The cic^{1} phenotype was rescued by a 9 kb transgene which expressed a transcript encoding an HMG-box transcription factor (Jiménez et al., 2000). The maternal and zygotic *fet* phenotypes were successfully rescued by a single copy of the same transgene. Rescued *fet* flies developed wild-type wings and produced eggs with wild-type dorsal appendages and hatching larvae.

We also tested the ability of *fet* to complement *bwk*. Maternal effect mutations in *bwk* cause abnormal dorsal appendages and bicaudal embryos with normal DV pattern (Rittenhouse and Berg, 1995); bwk^{8482} flies also show wing defects characterized by ectopic veins. *bwk* was shown to be required in the germline for normal eggshell and embryo patterning. P-element alleles of *bwk* complemented the

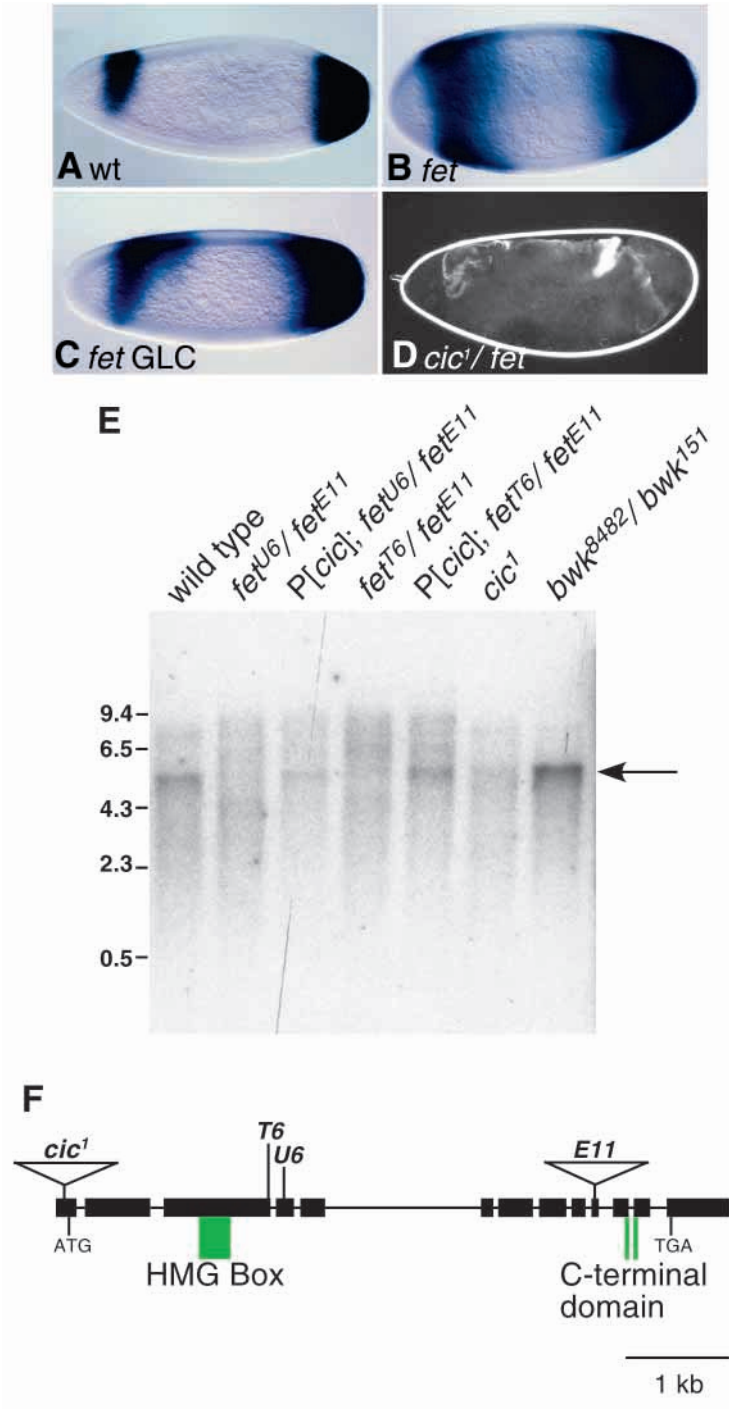

Fig. 6. *fet* is allelic to *cic*. (A-C) In the wild-type embryo (A), *tll* RNA expression is confined to the termini of the embryo. In embryos produced by fet^{T6}/fet^{E11} females (B) and fet^{E11} GLC embryos (C), *tll* RNA expression is derepressed towards the center of the embryo. (Lateral view, dorsal side upwards). (D) Cuticle of embryo produced by fet^{U6}/cic^{1} transheterozygote shows a *torso* gain-of-function phenotype. (E) RNA blot probed with *cic* cDNA probe. Each lane was loaded with 1 μg poly(A)$^+$ ovarian RNA isolated from females with the indicated genotypes. Mobilities of DNA markers are indicated on left. The arrow points to the 5.7 kb *cic* transcript. (F) The intron/exon organization of the *cic* transcript, with black boxes representing the 13 exons. The HMG box lies within exon 3, and the conserved C-terminal domain is located within exons 11 and 12. The positions of mutations fet^{T6}, fet^{U6}, and fet^{E11} are indicated above the boxes. The location of the cic^{1} *hobo* insertion has been described to be in the 5′ untranslated region (Jiménez et al., 2000).

fet eggshell and wing defects, while embryos laid by *fet/bwk* transheterozygotes were bicaudal (data not shown).

We analyzed transcripts expressed in the *cic/bwk* region on RNA blots (Fig. 6E). The level of a 5.7 kb transcript was reduced in both *fet* and cic^{1} ovaries. Expression of this transcript was restored in the ovaries of *fet* females carrying one copy of the transgene. The level of this 5.7 kb transcript was not affected in *bwk* mutant ovaries, and the *bwk* phenotype was not rescued by the 9 kb transgene (Jiménez et al., 2000).

Finally, we identified the lesions responsible for fet^{T6}, fet^{U6} and fet^{E11} in the open reading frame of the *cic* transcript (Fig. 6F). The *cic* gene (CG5067) contains 13 exons (Adams et al., 2000) (Fig. 6F), with the HMG box mapping to exon 3 and the conserved C-terminal domain being split between exons 11 and 12. The *T6* and *U6* mutations were each caused by a single nucleotide change in the coding region. For the *T6* allele, a G to A transition at position 2861 at the putative exon 3/intron 3 junction produces a S to G amino acid change, and, more importantly, is predicted to reduce splicing efficiency of intron 3, introducing an in-frame translational stop codon in the unspliced intron (see Materials and Methods). For the *U6* allele, a C to T transition at position 3007 produces an amber mutation in exon 4. For the *E11* allele, the P element was inserted with 3′ to 5′ orientation after nucleotide 6510 in exon 10, introducing an in-frame stop codon that is predicted to produce a truncated protein that lacks the conserved C-terminal domain.

Fet/Cic protein is expressed in follicle cell nuclei

After determining that *fet* and *cic* were allelic, we used anti-Cic antibodies (Jiménez et al., 2000) to visualize the distribution of Fet/Cic protein in the follicular epithelium. Cic expression appeared quite dynamic. In stage 9 egg chambers, Cic was expressed uniformly in follicle cell nuclei (data not shown); at stage 10A, Cic began to be downregulated on the dorsal side (Fig. 7A,C), and Cic was completely absent in dorsal follicle cells by stage 10B (Fig. 7B,D; see Discussion). As predicted from the preceding genetic analysis, Cic protein was expressed in cic^{1} ovaries (Fig. 7E) and absent in *fet* ovaries (Fig. 7F).

DISCUSSION

Establishment of DV polarity in the *Drosophila* egg requires *fet* activity during oogenesis in the follicle cells. Genetic analysis suggests that *fet* is required downstream of Egfr signaling, and acts in two different pathways to pattern the eggshell and embryo. We show that *fet* is allelic to the *cic* gene, which encodes an HMG-box transcription factor.

fet acts in separate pathways to establish DV polarity of the embryo and eggshell

Previous studies have described several dorsalizing mutations

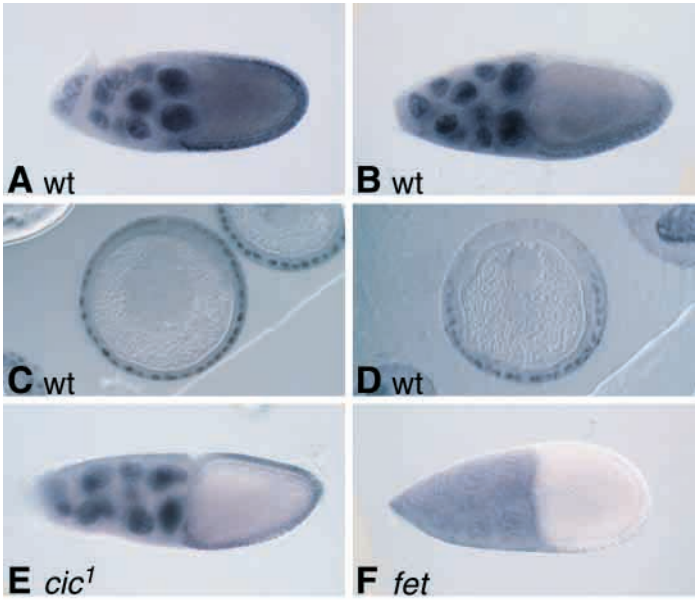

Fig. 7. Fet/Cic protein is expressed in follicle cell nuclei. (A,B) Stage 10A (A) and stage 10B (B) wild-type egg chamber showing expression of Fet/Cic protein in follicle cell nuclei (lateral view, dorsal side upwards). In the older chamber, Cic is downregulated on the dorsal side. (C,D) Egg chamber cross-section at the plane of the oocyte nucleus shows a subtle reduction of Cic protein on the dorsal side at stage 10A (C) and a complete absence of Cic on the dorsal side at stage 10B (D) (dorsal side upwards). (E,F) Cic protein is expressed in egg chambers from *cic*^{1} females (E) and absent in egg chambers from *fet*^{E11}/*fet*^{U6} females (F) (lateral view, dorsal side upwards).

that all affect some aspect of Egfr signaling, consistent with its role in coordinating DV polarity of the eggshell and embryo. Thus, it has been surprising to find that both the production of the Grk signal in the oocyte and initial activation of the Egfr in follicle cells appear unaffected in mutant *fet* ovaries. As a consequence of Egfr signaling, the follicular epithelium is normally partitioned into dorsal and ventral domains through the spatially restricted expression of *mirror* and *fringe* transcripts, respectively (Jordan et al., 2000; Zhao et al., 2000). Concomitant with this early response, Egfr modulates its own signaling during the course of oogenesis by inducing the expression of genes (e.g. *rho*, *kek1* and *Cbl*) that encode regulators, which act at the level of receptor activation. At early stage 10, which corresponds to the period when Egfr activation leads to the transcription of target genes, the expression patterns of *mirror*, *fringe*, *rho* and *kek1* (Fig. 3; data not shown) in mutant *fet* ovaries are indistinguishable from wild type.

The dorsalized phenotype observed with the loss of *fet* activity in follicle cells is apparently caused by a requirement for *fet* in each branch of the Egfr pathway that separately patterns the embryo and eggshell. In establishing DV polarity of the embryo, *fet* is required as an essential transcriptional regulator of *pipe* RNA expression in the ventral follicle cells (Fig. 5). By epistasis analysis, *fet* acts downstream of *Egfr* and causes a dorsalized phenotype even when Egfr signaling is reduced.

In establishing DV polarity of the eggshell, *fet* appears to be involved in refining Egfr activity later during oogenesis. Although *mirror-lacZ* is initially expressed correctly, the domain later expands around the anterior circumference of the egg chamber (Fig. 3I,J), suggesting a role for *fet* after the first round of Egfr signaling by Grk. This interpretation is supported by analysis of the double mutant. In contrast to the strictly linear relationship observed for establishing embryonic polarity, the eggshell phenotype produced by *Egfr*^{1}*; fet* females shows contributions from each of the individual phenotypes. The distance between dorsal appendages is reduced, as observed for the ventralizing *Egfr*^{1} mutation, but the individual appendage structure is broadened, as seen for the dorsalizing *fet* mutation.

In the wild-type eggshell, dorsal appendage pattern is achieved through refinement of the Egfr activation profile by both positive and negative feedback regulation (Freeman, 2000). Expression of *rhomboid* RNA is induced as a result of Egfr activation (Ruohola-Baker et al., 1993) and positively regulates continued Egfr signaling (Sapir et al., 1998). Induction of *argos* RNA expression leads to negative feedback on Egfr signaling at the dorsal midline, causing refinement of one domain of Egfr activation into two laterally symmetric domains that specify the placement of the paired dorsal appendages (Wasserman and Freeman, 1998).

This dorsal appendage pattern can be genetically altered in different ways. The *K10* and *squid* mutant phenotype is characterized by an eggshell with a fused cylindrical dorsal appendage around the anterior circumference of the egg deposited around a dorsalized embryo (Roth and Schüpbach, 1994). The *fet* and *Cbl* mutant eggshell phenotype appears distinct; rather than a single cylindrical structure, two laterally placed broadened dorsal appendages form, often associated with circumferential dorsal appendage base material. A change in either the strength or timing of Egfr signaling might translate into these different phenotypes.

Relationship between *fet* and *cic*

The *fet* mutations map to the *cic* transcript, which encodes an HMG-box transcription factor. Analysis of the *cic*^{1} maternal effect phenotype revealed two germline-encoded functions (Jiménez et al., 2000). The Cic protein acts as a repressor of *tll* transcription in the embryo termini and as a Dorsal-dependent repressor along the DV axis.

The experiments described show that establishment of DV polarity in the embryo requires maternal *cic* activity at two distinct stages. The characterization of the *fet* phenotype indicates that *cic* is first required during oogenesis to activate *pipe* transcription in the ventral follicle cells. The *cic*^{1} phenotype shows that *cic* is then required during embryogenesis as a co-repressor with Dorsal. In *cic*^{1} mutant embryos, *zen* RNA expression is expanded from its normal dorsally restricted domain to more lateral positions, although Twist expression appears wild type (Jiménez et al., 2000). By contrast, in *fet* mutant embryos, lack of *pipe* expression during oogenesis leads to a more severely dorsalized phenotype in the embryo, with *zen* RNA expressed circumferentially and Twist staining absent (Fig. 1).

In addition to the maternal effect eggshell and embryo phenotype, viable *fet* alleles exhibit wing phenotypes and strong *fet* alleles are lethal. This range of *fet* phenotypes, as compared with the *cic*^{1} phenotype, may be accounted for by

the molecular nature of the mutations. The cic^1 mutation is caused by a *hobo* mobile element insertion in the 5′ untranslated region of the *cic* transcript (Fig. 6F), while the bwk^{8482} allele, which also has a maternal effect phenotype caused by a germline defect, is associated with a P-element insertion in the same region (Jiménez et al., 2000). These mobile elements may contain cryptic promoters which allow sufficient expression of the *cic* transcript to rescue the somatic but not the germline functions of this gene (Kiger et al., 1999). The analysis of *fet* mutations, which alter the coding region of the *cic* transcript, suggests that *cic* is required in a number of signaling pathways during *Drosophila* development.

Mechanism of *cic* action in follicle cells

In regulating terminal development in the embryo, *cic* acts by repressing *tll* transcription (Jiménez et al., 2000). In controlling DV asymmetry of the follicular epithelium, two models of *cic* action are formally possible: either *cic* represses an unknown negative regulator of *pipe* expression or *cic* positively regulates *pipe*. *cic* is unlikely to be a direct negative regulator of *pipe* transcription, as *pipe* expression is abolished in *fet* mutant ovaries. However, *cic* also acts in the follicle cells in the eggshell patterning pathway, where it is possible that *cic* acts as a negative regulator at the *mirror* promoter, because ectopic *mirror* expression is observed in *fet* mutants at later stages of oogenesis.

In the blastoderm embryo, the receptor tyrosine kinase (RTK) Torso positively regulates *tll* expression at the termini by repressing *cic* action, resulting in the absence of Cic protein at the termini; uniform distribution is observed in embryos produced by $torso^-$ females (Jiménez et al., 2000). In an intriguing parallel, Cic expression in the follicular epithelium is absent from the dorsal side; uniform distribution is observed in grk^- ovaries (data not shown), suggesting that this downregulation could be a consequence of signaling by the RTK Egfr. However, Cic asymmetry is detected after the initiation of *pipe* transcription. Thus, the pattern of *pipe* transcription cannot be determined by the physical distribution of Cic protein, although it remains possible that Cic is inactivated as a result of Egfr signaling prior to its disappearance from dorsal follicle cells.

Cic shows features of a family of HMG-box proteins that bind DNA in a sequence-specific manner, although DNA-binding activity has not been detected yet (Jiménez et al., 2000). As shown for the case of *Drosophila* TCF (Cavallo et al., 1998), a target of the *wingless* signaling pathway, some HMG-box proteins can act as positive or negative transcriptional regulators, depending on promoter contexts and interaction with co-factors. As positive regulators of transcription, HMG-box proteins bind in the minor groove of DNA and induce bending, which can bring distal regulatory elements closer to promoters to enhance transcription. As negative regulators of transcription, HMG-box proteins interact with co-repressors, such as Groucho (Gro). Gro does not bind to DNA directly, but rather is recruited to the template by DNA-bound transcription factors (Fisher and Caudy, 1998; Parkhurst, 1998; Chen and Courey, 2000). Repression probably occurs by several mechanisms, including recruitment of the histone deacetylase Rpd3 (Chen et al., 1999), which presumably silences transcription by altering local chromatin structure.

Cic and Gro have been shown to interact in vitro (Jiménez et al., 2000). For the Torso pathway, the biological significance of this physical association is supported by the analysis of *gro* germline clones, which show mutant phenotypes that are identical to those caused by the germline cic^1 defects (Paroush et al., 1994; Paroush et al., 1997). It remains to be determined whether recruitment of Gro co-repressor by Cic is conserved in follicle cells and the wing disc.

D. M. acknowledges the guidance of K. V. Anderson in the nascent stages of this work. We are grateful to C. Berg and J. Casanova for generously sharing information and reagents. We also thank D. Bilder for advice on making ovarian clones, M. Walczak for assistance in generating *dec*-marked clones, and W. Bender, G. Denton and C. Hashimoto for their helpful suggestions on the manuscript. This work was funded by grant GM52084 (awarded to D. M.) from the NIH; D. J. G. was supported by postdoctoral fellowship PF4443 from the American Cancer Society.

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