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Throughout this article and in the references, Trudi Schüpbach's name appeared incorrectly as Schübach.

We apologize to the authors and readers for this mistake.

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Capicua regulates follicle cell fate in the *Drosophila* ovary through repression of *mirror*

Matthew R. Atkey, Jean-François Boisclair Lachance, Monica Walczak, Tahilia Rebello and Laura A. Nilson*

The dorsoventral axis of the *Drosophila* egg is established by dorsally localized activation of the epidermal growth factor receptor (Egfr) in the ovarian follicular epithelium. Subsequent positive- and negative-feedback regulation generates two dorsolateral follicle cell primordia that will produce the eggshell appendages. A dorsal midline domain of low Egfr activity between the appendage primordia defines their dorsal boundary, but little is known about the mechanisms that establish their ventral limit. We demonstrate that the transcriptional repressor Capicua is required cell autonomously in ventral and lateral follicle cells to repress dorsal fates, and functions in this process through the repression of *mirror*. Interestingly, ectopic expression of *mirror* in the absence of *capicua* is observed only in the anterior half of the epithelium. We propose that Capicua regulates the pattern of follicle cell fates along the dorsoventral axis by blocking the induction of appendage determinants, such as *mirror*, by anterior positional cues.

KEY WORDS: *Drosophila*, *capicua*, *mirror*, Follicle cells, Patterning

INTRODUCTION

Epithelial patterning during development involves the generation, transmission and interpretation of positional cues. In the egg chambers of the *Drosophila* ovary, the follicular epithelium is patterned through a series of localized inductive interactions with the underlying germline (Nilson and Schübach, 1999; Roth, 2003; Van Buskirk and Schübach, 1999). At the end of oogenesis, the follicle cells secrete the eggshell, which displays pronounced axial asymmetries and locally specialized structures that represent the outcome of the patterning process (Waring, 2000). For example, the anterior eggshell exhibits two prominent appendages flanking the dorsal midline, reflecting a complex pattern of follicle cell fates along the dorsoventral (DV) axis. Defects in epithelial patterning can therefore be recognized through the resulting eggshell abnormalities.

DV asymmetry within the follicular epithelium is established during mid-oogenesis by the product of the *gurken* (*grk*) gene, a transforming growth factor (TGF) α-like molecule restricted to the future dorsal side of the oocyte (Neuman-Silberberg and Schübach, 1993; Neuman-Silberberg and Schübach, 1996). Grk acts as a localized ligand for the *Drosophila* epidermal growth factor receptor (Egfr) in the overlying follicle cells, and the resulting localized Egfr activity defines the dorsal region of the epithelium (Nilson and Schübach, 1999; Roth, 2003; Van Buskirk and Schübach, 1999). As oogenesis proceeds, this initial distinction of dorsal from ventral follicle cells is refined to generate a more complex pattern of cell fates, including two dorsolateral appendage primordia separated by a population of dorsal midline cells. The resolution of primordia and midline fates within the dorsal domain is achieved through feedback modulation of Egfr activity. Gurken-Egfr signaling induces expression of *rhomboid* (*rho*), which leads to autocrine production of the active form of another Egfr ligand, Spitz (Spi), presumably resulting in amplification of the initial signal. Egfr activation also induces expression of Argos (Aos), a direct antagonist of Spi, in the dorsal-most follicle cells, thereby resolving the initial broad region of Egfr signaling into two dorsolateral peaks (Klein et al., 2004; Peri et al., 1999; Shvartsman et al., 2002; Wasserman and Freeman, 1998). The position of the primordia is also regulated along the anteroposterior (AP) axis by Decapentaplegic (Dpp), a member of the Tgfβ family of signaling molecules, produced by the anterior-most follicle cells (Deng and Bownes, 1997; Peri and Roth, 2000; Twombly et al., 1996).

The dorsal midline domain between the appendage primordia defines their dorsal boundary, but how the ventral boundary of each primordium is determined is not understood. Computational models of Aos function in the embryonic ventral ectoderm have suggested that Aos may act at a short range to sequester Spi and restrict its diffusion (Klein et al., 2004; Reeves et al., 2005). However, a ventral expansion of appendage-producing follicle fates is not observed upon loss of *aos* function (Wasserman and Freeman, 1998), indicating that the ventral limit of the appendage primordia is not defined by Aos-mediated regulation of Spi distribution. Alternatively, a graded dorsal distribution of Egfr ligand could define the ventral limit of the primordia, establishing dorsal fates only where levels exceed a critical threshold. However, it is not clear whether additional factors function to translate this potentially graded signal into a sharp boundary between primordium and non-primordium fates (Shilo, 2005). Moreover, how the dorsal patterning process is integrated with anterior positional information, which is present throughout the DV axis, is not understood.

DV patterning of the follicular epithelium requires *capicua* (*cic*) function. *cic* mutant females produce 'dorsalized' eggs that exhibit a ventral expansion of dorsal pattern elements. Although similar eggshell phenotypes can result from the mislocalization of Grk (Neuman-Silberberg and Schübach, 1993; Neuman-Silberberg and Schübach, 1996), the expression and distribution of *grk* are unaffected in *cic* mutant ovaries, suggesting that *cic* acts downstream of *grk* (Goff et al., 2001). Dorsalization can also result from increased Egfr levels and signaling, as observed upon loss of the D-Cbl (Cbl – FlyBase) ubiquitin-protein ligase in the follicle cells (Pai et al., 2000). However, a general expansion of Egfr target gene expression is not observed in *cic* mutant ovaries (Goff et al., 2001), indicating that the *cic* eggshell phenotype does not result

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from generally increased Egfr activity. Loss of *cic* therefore has a unique effect on eggshell patterning, suggesting that Cic functions at a novel step in the regulation of Egfr-mediated cell fate determination.

cic encodes an evolutionarily conserved HMG-box transcription factor that functions as a transcriptional repressor during embryogenesis and wing development (Jimenez et al., 2000; Roch et al., 2002). In general, downregulation of Cic levels through receptor tyrosine kinase (RTK) signaling activates gene expression. For example, downregulation of Cic by Torso activity at the poles of the early embryo establishes the embryonic termini, and loss of Cic in the wing imaginal disk through localized Egfr signaling specifies the presumptive wing vein tissue (Jimenez et al., 2000; Roch et al., 2002). In the ovary, Cic is downregulated in dorsal follicle cells during DV patterning (Goff et al., 2001), suggesting that Cic could negatively regulate RTK target genes in this tissue as well.

We show that *cic* is required cell autonomously in ventral and lateral follicle cells to repress appendage-producing fates, and that this fate transformation correlates with and depends on the ectopic expression of *mirror* (*mirr*), an Egfr target normally expressed in dorsal anterior follicle cells. We further demonstrate that the ectopic expression of Mirr, a homeodomain transcription factor, at any position within the follicular epithelium is sufficient to determine appendage-producing fate. Interestingly, the ectopic *mirr* expression and appendage-producing fates observed in *cic* mutant ovaries are restricted to the anterior half of the follicular epithelium. Collectively, these data suggest that Cic regulates the specification of the appendage primordia by inhibiting the induction of dorsal fate determinants by anterior positional cues.

MATERIALS AND METHODS

Drosophila *strains*

The following *Drosophila* stocks were used: Oregon R; cic^{fetU6}, cic^{fetT6}, cic^{fetE11} (Goff et al., 2001), cic^{BA53}, $mirr^{e48}$ (McNeill et al., 1997), *P{lacW}l(3)mirr*^{6D1} (*mirr-lacZ*) (Ruohola-Baker et al., 1993), fng^{13} (Grammont and Irvine, 2001), *UAS-mirr* (McNeill et al., 1997), *P{lacW}kek1*^{AN296} (Sapir et al., 1998), grk^{HK}, grk^{2B6} (Thio et al., 2000), *rhomboid*^{7M43} and *rhomboid*^{6} (Wasserman et al., 2000). Loss of function clones were generated using *y w P{hsFLP}122; P{Ubi-GFPnls} P{neoFRT}82B* or *y w P{hsFLP}122; P{hs-N-myc} P{neoFRT}82B*. The stock *y w; P{GAL4-Act5C(FRT.CD2).P}S, P{UAS-GFP}* was used to generate gain-of-function clones.

Sequencing of the cicBA53 allele

The cic^{BA53} allele was identified in a genetic screen for patterning defects associated with follicle cell clones. Overlapping fragments of approximately 800 base pairs each from the *cic* locus were amplified by PCR from genomic DNA of homozygous cic^{BA53} embryos, and from flies homozygous for the parental chromosome on which the cic^{BA53} mutation was induced, and then sequenced by the Genome Québec Innovation Centre (Montréal, QC). The only sequence change was a base pair transition from C to T at position 2563 of the genomic sequence (with position 1 being the first base of the 5′ UTR), which introduces a stop codon near the end of the third exon.

Generation of mosaic ovaries

Loss-of-function follicle cell clones were generated by FLP-FRT-mediated mitotic recombination as described, except that recombination was induced in adults (Caceres and Nilson, 2005; Xu and Rubin, 1993). Homozygous mutant clones were marked by the absence of nuclear green fluorescent protein (GFP) or N-Myc (Xu and Rubin, 1993). To generate GFP-marked gain-of-function clones, females bearing the *y w P{hsFlp}122* chromosome, the Flp-out cassette *P{GAL4-Act5C(FRT.CD2).P}S, P{UAS-GFP}* (Pignoni and Zipursky, 1997), and a gene of interest under UAS control were incubated at 37°C for 2 minutes and 15 seconds.

Immunohistochemistry

Immunohistochemistry was performed as described previously (Caceres and Nilson, 2005), except incubation with secondary antibodies was performed overnight at 4°C. The following primary antibodies were used, each at a 1:50 dilution: anti-BR-C (25E9, gift of Greg Guild); anti-β-galactosidase (40-1a, Developmental Studies Hybridoma Bank); anti-DE-Cadherin (DCAD2, gift of Tadashi Uemura, Kyoto University); anti-c-Myc (A-14, Santa Cruz Biotechnology). Secondary antibodies (Molecular Probes) were used at the following dilutions: goat anti-mouse Alexa Fluor 568, 1:500; goat anti-rabbit Alexa Fluor, 1:800; goat anti-rat Alexa Fluor 546, 1:800.

RESULTS

Homozygous *cic* mutant females produce dorsalized eggs

The DV pattern of the wild-type *Drosophila* eggshell is distinguished by its overall shape and by two prominent appendages flanking the dorsal midline near the anterior of the eggshell (Fig. 1A). Females homozygous for viable combinations of *cic* alleles are sterile, and produce eggs with an expanded dorsal midline region and broad, laterally shifted appendages. These eggs also typically exhibit a ‘collar’ of ectopic appendage-like material around the ventral anterior circumference (Fig. 1B-F) (Goff et al., 2001). The dorsalmost region remains free of appendage material (see Fig. 1D).

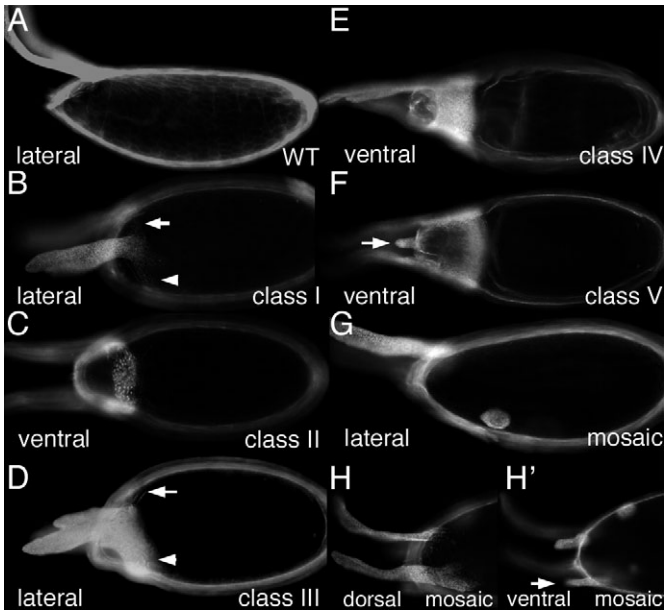

Fig. 1. *cic* mutant females produce eggs with a range of dorsalized phenotypes. (**A**) Wild-type egg (lateral view), exhibiting two dorsal appendages separated by an appendage-free dorsal midline region. (**B-F**) Eggs from *cic* mutant females, ventral or lateral views, as indicated. Eggs are grouped into five classes according to phenotypic severity. (B) Weak *cic* eggshell phenotype (lateral view). The dorsal midline region is expanded (arrow) and the appendages are broader and positioned laterally with a trace of ventral collar material (arrowhead). (C) Similar to B, but exhibiting a ventral collar of ectopic appendage-like material. (D) Egg with a thicker ventral collar (arrowhead). Lateral view illustrates that the collar is strictly ventrolateral and does not extend to the dorsal side (arrow). (E) Collar is more pronounced than in D. (F) Similar to E, with small anterior projections. (**G-H’**) Eggs from females with follicle cell clones homozygous for cic^{fetU6}. (G) Foci of ectopic appendage material are observed in the ventral and lateral anterior region. (H,H′) At the extreme anterior, ectopic appendage-like projections can be observed (arrow). H is a dorsal view, H′ a ventral view.

Table 1. Dominant suppression of *cic* eggshell phenotypes by reduction of *mirr*

		Eggshell phenotype (%)				
	n	I	II	III	IV	V
cic^{BA53}/cic^{fetU6}	64	0	0	0	14.1	85.9
cic^{fetT6}/cic^{fetU6}	177	0	32.8	44.6	20.9	1.7
cic^{fetE11}/cic^{fetU6}	140	12.9	19.3	37.9	30.0	0
$cic^{BA53}/mirr^{l(3)6D1}$ cic^{fetU6}	184	0	0.5	34.8	32.1	32.6
$cic^{fetT6}/mirr^{l(3)6D1}$ cic^{fetU6}	202	0.5	42.6	42.1	14.9	0
$cic^{fetE11}/mirr^{l(3)6D1}$ cic^{fetU6}	163	34.4	37.4	24.5	3.7	0

Eggs from females of the indicated genotypes were classified into the phenotypic categories illustrated in Fig. 1.

We defined five phenotypic classes to describe the range of eggshell phenotypes observed, which seem to increase in severity with the thickness of the ventral collar.

The severity of the phenotype depends on the allelic combination. Eggs from cic^{BA53}/cic^{fetU6} females fall into the strongest phenotypic categories, those from cic^{fetT6}/cic^{fetU6} females fall mostly into the middle categories, whereas those from cic^{fetE11}/cic^{fetU6} females are distributed across the weaker categories (Table 1). This interpretation of phenotypic severity is consistent with the molecular nature of these alleles. cic^{T6} and cic^{U6} are predicted to produce proteins truncated just downstream of the HMG box, whereas the cic^{E11} allele is the result of a P-element insertion much further downstream, just before a conserved C-terminal domain (Goff et al., 2001). cic^{BA53} is a novel allele with a premature stop codon upstream of the cic^{T6} and cic^{U6} mutations and is therefore predicted to produce the most severely truncated protein (see Materials and methods).

cic mutant clones are associated with ectopic dorsal appendage material

To determine the effect of a localized loss of *cic* function, we used the Flp/FRT system (Xu and Rubin, 1993) to generate follicle cell clones homozygous for the cic^{fetU6} allele. Eggs from mosaic females exhibited ventral and lateral foci of ectopic appendage-like material (Fig. 1G-H′), and analysis of follicle cell clones marked with the *defective chorion 1* eggshell marker (Nilson and Schübach, 1998) confirmed that these foci were associated with ventral and lateral clones of *cic* mutant follicle cells (data not shown). Ectopic appendage material was not observed on the dorsal side. This mosaic analysis indicates that *cic* is required in the follicle cells for eggshell patterning. This ectopic appendage material was restricted to the anterior half of the eggshell, while posterior clones exhibited no detectable phenotype (data not shown), consistent with previous observations that only anterior follicle cells are competent for appendage production (Peri and Roth, 2000; Queenan et al., 1997). Interestingly, ectopic appendage material at the extreme anterior of the eggshell often appears as appendage-like projections (Fig. 1H′), suggesting that only *cic* mutant follicle cells in the corresponding region of the columnar follicular epithelium can form an appendage-producing epithelial tube.

Mutant eggshell phenotypes reflect a change in follicle cell fate

The ventral expansion of dorsal eggshell structures suggests a corresponding shift in the DV pattern of follicle cell fates. To visualize follicle cell fate directly, we examined the expression of the *Broad-Complex* (*BR-C*) locus, which encodes a group of four alternatively spliced zinc finger transcription factor isoforms whose elevated expression after stage 10 of oogenesis marks the dorsal appendage primordia (Bayer et al., 1996; Deng and Bownes, 1997; DiBello et al., 1991; Tzolovsky et al., 1999). In wild-type ovaries, BR-C is first detected at uniform levels in all follicle cells, but beginning at stage 10 BR-C expression is lost from dorsal anterior and dorsal midline follicle cells, and then becomes elevated in two populations of anterior follicle cells flanking the dorsal midline (Fig. 2A,B). These cells comprise the majority of each primordium and

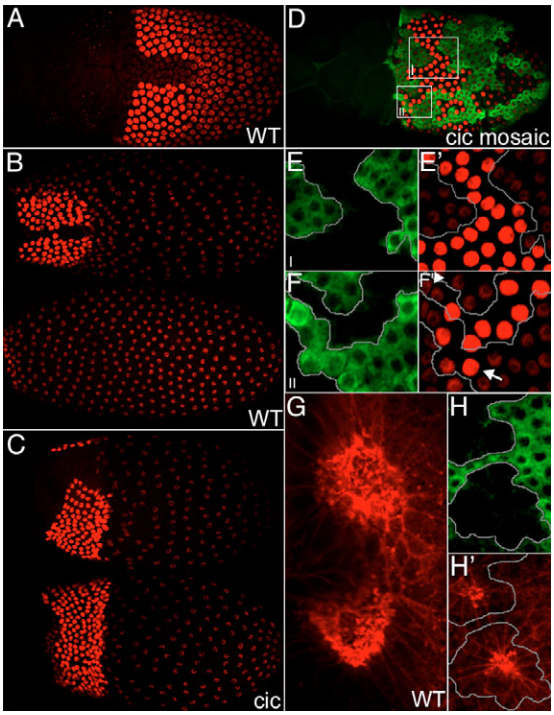

Fig. 2. Ventral anterior follicle cells adopt an appendage-producing fate in *cic* mutant egg chambers. BR-C expression was visualized in wild-type (A,B) and *cic* mutant (C-F) egg chambers using an antibody recognizing all BR-C isoforms (Emery et al., 1994). (**A**) Wild-type stage 10 egg chamber (dorsal view). (**B**) Wild type, stage 12, dorsal (top) and ventral (bottom) views. (**C**) Stage 12 cic^{fetU6}/cic^{fetT6} egg chambers, dorsolateral (top) and ventrolateral (bottom) views. The high BR-C nuclei are more densely organized than those with low BR-C. (**D**) Stage 10B egg chamber (ventral view) with homozygous cic^{fetU6} follicle cell clones marked by the absence of N-Myc (green). (**E**,**E′**) Close-up of box I in D; clone boundary outlined in gray. In the vast majority of clones, ectopic elevated BR-C is cell autonomous. (**F**,**F′**) Close-up of box II in D. Rare cases of nonautonomy (arrow, arrowhead) were observed near the endogenous appendage primordia. (**G**) Wild type, stage 12 (dorsal view), stained with rhodamine-labeled phalloidin. The cells of the appendage primordia are constricted apically. (**H**) Stage 12 ventral anterior cic^{fetU6} follicle cell clones marked by the absence of N-Myc. (**H′**) Rhodamine phalloidin staining of the clones in H reveals similar cell shape changes in *cic* mutant cells.

will form the roofs of the epithelial tubes that secrete the appendages (Dorman et al., 2004). The remaining posterior and ventral cells exhibit low BR-C levels, which diminish as oogenesis proceeds.

In ovaries from *cic* homozygotes, BR-C expression is normal until stage 10A (data not shown). Subsequently, however, high BR-C levels are expanded throughout the ventral and lateral anterior circumference of the follicular epithelium (Fig. 2C). This ventral expansion of high BR-C levels is consistent with the *cic* eggshell phenotype and confirms that the collar of ventral eggshell material reflects the ectopic induction of an appendage-producing follicle cell fate. Notably, BR-C expression is still lost from the dorsal midline follicle cells in *cic* mutant egg chambers, indicating that *cic* is not required for the refinement of dorsal follicle cell pattern that resolves the two appendage primordia.

Ectopic high BR-C levels were also observed after stage 10A in *cic* mutant follicle cell clones (Fig. 2D-F). Consistent with the mosaic eggshell phenotype, this effect was restricted to cells in the anterior half of the columnar epithelium (Fig. 2D). Dorsal clones had no effect on the BR-C expression pattern (data not shown), consistent with the lack of Cic in this domain at this stage (Goff et al., 2001). In the vast majority of clones, the ectopic expression of high BR-C levels was cell autonomous (Fig. 2E), although we did observe rare cases of nonautonomy, where high BR-C levels were also observed in a few adjacent wild-type cells (Fig. 2F), typically near the endogenous appendage primordia.

In addition to ectopic expression of BR-C, loss of Cic results in morphological changes characteristic of appendage-producing follicle cells. In wild-type egg chambers, appendage morphogenesis begins with the apical constriction of the high BR-C expressing cells of the appendage primordia (Fig. 2G) (Dorman et al., 2004). With the exception of those at the dorsal midline, *cic* mutant follicle cells undergo apical constriction reminiscent of that observed in the endogenous appendage-producing populations (Fig. 2H). As observed for ectopic high BR-C levels, this effect is restricted to *cic* mutant follicle cells in the anterior half of the epithelium. In addition, the follicle cell nuclei appear more closely packed in both the endogenous appendage roof primordia and in regions of anterior *cic* mutant cells, presumably as a consequence of this apical constriction (Fig. 2B,C). These molecular and morphological data indicate that *cic* mutant follicle cells within the ventral and lateral anterior region of the follicular epithelium adopt an appendage-producing follicle cell fate, suggesting that Cic normally functions cell autonomously to repress this fate.

cic mutant follicle cells express ectopic *mirr*

Initial characterization of Egfr target gene expression in *cic* mutant egg chambers identified *mirr*, a member of the *Iroquois-Complex* (*Iro-C*) of genes encoding homeodomain-containing transcription factors, as a possible regulatory target of Cic (Goff et al., 2001; McNeill et al., 1997). In midoogenesis, *mirr* is expressed in dorsal anterior follicle cells and this expression depends on Egfr signaling (Jordan et al., 2000; Zhao et al., 2000b). Disruption of *mirr* function has been associated with dorsal appendage defects, but the function of *mirr* in follicle cell fate determination is not well understood.

We used a *mirr-lacZ* transgenic reporter (Jordan et al., 2000; Ruohola-Baker et al., 1993) to characterize *mirr* expression in wild-type and *cic* mutant egg chambers. In wild-type egg chambers, *mirr* expression is restricted to dorsal anterior follicle cells, and the boundary of this domain aligns with that of the high BR-C domain (Fig. 3A,A′). In *cic* mutants, the dorsal anterior *mirr* expression pattern is established normally, but, by stage 10B, *mirr* expression expands ventrally and is detected throughout the DV circumference of the epithelium, although dorsal levels of *mirr* appear to be consistently higher at this stage (Fig. 3B) (Goff et al., 2001). Similar to the wild-type pattern, this ectopic *mirr* expression is restricted to the anterior portion of the epithelium.

Clones of *cic* mutant follicle cells also exhibit ectopic *mirr* expression, indicating that the regulation of *mirr* requires *cic* function in the follicle cells (Fig. 3C-E′). Consistent with the *cic* homozygous phenotype, this effect differs along the AP axis; ectopic *mirr* expression is observed in *cic* mutant follicle cells in roughly the anterior half of the columnar epithelium (Fig. 3C,C′, arrowheads), but not in those in the posterior half (Fig. 3C,C′, arrows). Although ectopic *mirr* expression may appear to extend further to the posterior

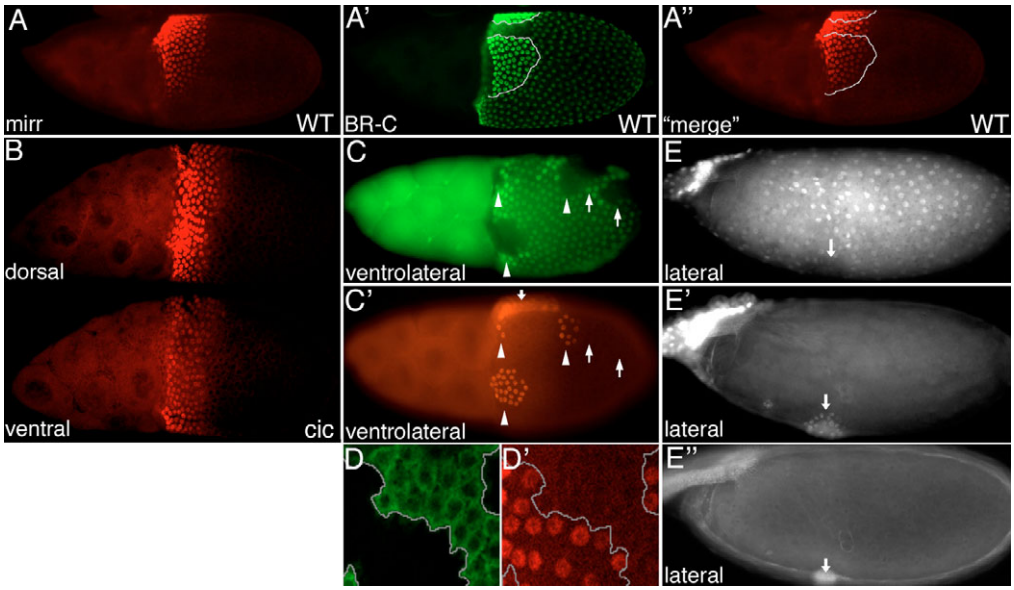

Fig. 3. *cic* mutant follicle cells exhibit ectopic *mirr* expression. (**A-A″**) Wild-type stage 10B egg chamber (dorsolateral view). (A) *mirr-lacZ* is expressed in dorsal anterior follicle cells. (A′) At this stage, the two dorsal anterior high BR-C domains (outlined) have been established. (A″) Overlay illustrates that the *mirr-lacZ* domain aligns with the high BR-C domains. (**B**) Stage 10B *cic* mutant egg chamber, dorsal (top) and ventral (bottom) views. (**C**) Stage 10B (ventrolateral view). Homozygous cic^{fetU6} follicle cell clones are marked by the absence of nuclear GFP. (**C′**) Endogenous dorsal anterior (top arrow) and ectopic anterior *mirr-lacZ* expression in the egg chamber in C. Ectopic *mirr-lacZ* is observed in cells in the anterior half (arrowheads), but not in the posterior half (arrows), of the columnar epithelium. (**D**) Ventral cic^{fetU6} mutant follicle cell clone (stage 10B) marked by the absence of N-Myc. (**D′**) Ectopic *mirr-lacZ* expression is cell autonomous. (**E-E″**) Stage 14 egg chamber with a ventral cic^{fetU6} mutant clone (E, arrow) exhibiting ectopic *mirr-lacZ* expression (E′, arrow) and ectopic appendage material (E″, arrow).

in mosaics than in homozygous epithelia (compare Fig. 3B with 3C′), this apparent difference can be attributed to the morphological changes in the follicle cells in the homozygotes, which result in the clustering of their nuclei and shift their apparent position anteriorly (see also Fig. 2C). Although ectopic *mirr* was detected in a few wild-type cells adjacent to rare clones near the endogenous *mirr* domain, the ectopic expression of *mirr-lacZ* in the vast majority of *cic* mutant clones was cell autonomous (Fig. 3C-D′). In late-stage egg chambers, these *mirr*-expressing cells surround foci of ectopic appendage material (Fig. 3E-E″), further supporting a correlation between *mirr* expression and the determination of appendage-producing fate.

Reduction of *mirr* function suppresses the *cic* phenotype

The correlation between loss of *cic* function and ectopic *mirr* expression suggested that deregulation of *mirr* may contribute to the patterning defects in *cic* mutant egg chambers. To test this hypothesis, we asked whether a reduction in *mirr* function would suppress the *cic* eggshell phenotype. For all combinations of *cic* alleles tested, heterozygosity for a hypomorphic *mirr* allele (Jordan et al., 2000; Ruohola-Baker et al., 1993) resulted in greater percentages of eggs falling into weaker phenotypic categories (Table 1). For cic^{fetU6}/cic^{BA53} and cic^{fetU6}/cic^{fetE11}, these shifts were highly statistically significant (χ^2-tests, $P<0.0001$). Moreover, *mirr* mutant follicle cell clones in a homozygous *cic* mutant background do not exhibit the high BR-C levels characteristic of the cell fate transformation observed in *cic* mutant egg chambers. This effect is cell autonomous (Fig. 4A,A′). The suppression of the *cic* phenotype by a loss of *mirr* function indicates that deregulation of *mirr* expression underlies the *cic* phenotype and suggests that Mirr is an important determinant of appendage-producing fate.

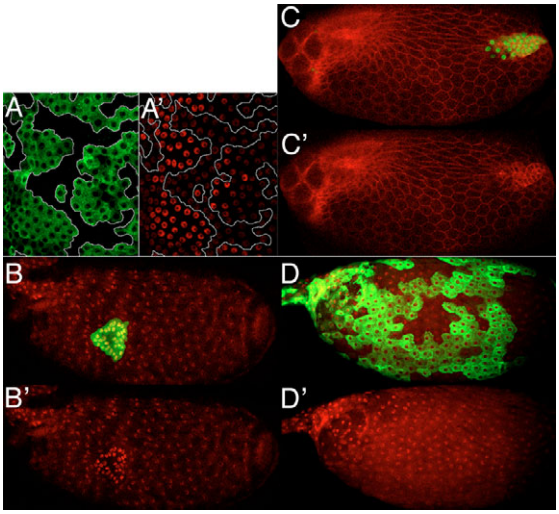

Fig. 4. Ectopic *mirr* expression is necessary and sufficient to induce appendage-producing fate. (**A**,**A′**) Ventral anterior region of stage 11 cic^{fetT6}/cic^{fetU6} egg chamber bearing follicle cell clones homozygous for $mirr^{e48}$ (A, absence of green). Ectopic high BR-C expression (A′, red) is abolished in the *mirr* mutant cells. (**B**,**B′**) Stage 14 egg chamber (lateral view) with follicle cell clones expressing UAS-*mirr* positively marked with GFP (green). BR-C expression (red) is also shown. (**C**,**C′**) Stage 12 egg chamber (dorsolateral view) with positively-marked follicle cell clones expressing UAS-*mirr* (green). DE-Cadherin expression (red) was visualized with an anti-DE-cadherin antibody. (**D**,**D′**) Stage 14 egg chamber (ventrolateral view) with homozygous fng^{13} follicle cell clones (D, absence of green). BR-C expression is shown in red.

Ectopic expression of *mirr* is sufficient to induce features of appendage-producing fate

To determine whether ectopic *mirr* expression is sufficient to induce this appendage-producing fate, we used the Flip-out/GAL4 technique to generate GFP-marked clones expressing UAS-*mirr* (Pignoni and Zipursky, 1997; Yang et al., 1999). Follicle cells expressing ectopic *mirr* exhibit elevated levels of BR-C (Fig. 4B,B′), and display the apical constriction and nuclear organization characteristic of the endogenous appendage primordia and *cic* mutant follicle cells in the anterior half of the epithelium (Fig. 4C,C′; data not shown). In later stages, these cells were associated with ectopic appendage material (data not shown). These data indicate that expression of *mirr* is sufficient to determine multiple characteristics of appendage-producing fate. This effect is less dramatic than that observed in *cic* mutant cells, however, and is likely to reflect either lower levels of *mirr* expression driven by the UAS element, or a contribution of other Cic targets. Interestingly, although the cell fate transformation resulting from loss of *cic* function was confined to the anterior half of the epithelium, the fate changes associated with UAS-*mirr* expression were observed even in posterior clones (Fig. 4C,C′), suggesting that the competence to adopt this fate is restricted at the level of *mirr* regulation.

A *fng* expression boundary is not sufficient for induction of appendage-producing fate

Our data implicate *mirr* as an important Cic target and appendage-producing fate determinant. One candidate regulatory target of Mirr in this process is *fringe* (*fng*), which encodes a glycosyltransferase that regulates cell fate decisions through the modulation of Notch signaling (Blair, 2000; Bruckner et al., 2000; Hicks et al., 2000; Moloney et al., 2000). Mirr regulates the expression of *fng* in a variety of developmental contexts. For example, repression of *fng* by Mirr in the *Drosophila* eye imaginal disc establishes a *fng* expression boundary, which in turn generates a center of Notch signaling activity that defines the equator of ommatidial polarity (Cho and Choi, 1998; Dominguez and de Celis, 1998; Papayannopoulos et al., 1998; Yang et al., 1999). Mirr also represses *fng* in the ovary, but a function for the dorsal anterior *fng* expression boundary in midoogenesis has not been demonstrated (James et al., 2002; Jordan et al., 2000; Peri et al., 2002; Zhao et al., 2000b).

Given our observation that ectopic *mirr* is sufficient to determine an appendage-producing fate, we hypothesized that Mirr may induce this fate through the repression of *fng* and the creation of a boundary of *fng* expression. To test this idea, we generated *fng* mutant follicle cell clones, reasoning that the resulting ectopic *fng* expression boundaries should be equivalent to those resulting from ectopic *mirr* expression and might therefore induce the same change in cell fate. Although *fng* clones at the egg chamber termini result in defective egg chambers that degenerate in midoogenesis, clones in the main body of the follicular epithelium do not affect egg chamber development and can be analyzed for patterning defects (Grammont and Irvine, 2001; Grammont and Irvine, 2002; Zhao et al., 2000b). Ventral and lateral *fng* clones do not exhibit high BR-C levels, changes in cell shape, or production of ectopic appendage material (Fig. 4D,D′; data not shown), indicating that a *fng* expression boundary is not sufficient to induce an appendage-producing fate and that *mirr* acts through other targets in this cell fate decision.

Ectopic *mirr* expression in *cic* mutant egg chambers is independent of *grk* and *rhomboid*

Although the localization of Grk restricts high levels of Egfr activity to the dorsal side of the egg chamber, low levels of Egfr ligand are also present ventrally, as revealed by the observation that loss of the ubiquitin ligase Cbl from ventral follicle cells results in *grk*-dependent ventral Egfr activity (Pai et al., 2000). As dorsal anterior expression of *mirr* during DV patterning requires Grk-Egfr signaling (Jordan et al., 2000; Zhao et al., 2000b), we considered the possibility that the ectopic expression of *mirr* in the absence of *cic* might result from an increased sensitivity of follicle cells to these low levels of ventral Egfr activity. To test this hypothesis, we asked whether loss of *grk* function could suppress the ectopic expression of *mirr* in *cic* mutant egg chambers.

Control egg chambers from *grk* mutant females that were also heterozygous for *cic* exhibited no *mirr* expression in dorsal anterior follicle cells, consistent with previous observations (Jordan et al., 2000; Zhao et al., 2000b) and confirming that this allelic combination eliminates *grk* function (Fig. 5B). In egg chambers that were homozygous for mutant alleles of both *cic* and *grk*, the high dorsal levels of *mirr* observed in *cic* mutant egg chambers (Fig. 5A) were abolished, but ectopic *mirr* expression was still detected throughout the anterior circumference (Fig. 5D,D′). Consistent with this *mirr* expression pattern, the resulting eggshells exhibited the loss of appendages characteristic of loss of *grk* function (Fig. 5C) but retained a pronounced collar of appendage material (Fig. 5E). These data indicate that, although the dorsal high point of *mirr* expression requires *grk*, the ectopic *mirr* expression resulting from the loss of *cic* is *grk* independent.

We also investigated whether the deregulation of *rhomboid* contributes to the *cic* mutant phenotype. Because Rhomboid generates the active form of the Egfr ligand Spi, ectopic *rhomboid* could potentially lead to ectopic Spi-mediated Egfr activation, which could in turn account for the ectopic *mirr* expression in *cic* mutant ovaries. In fact, ectopic expression of *rhomboid* throughout the follicular epithelium can result in dorsalized eggs (Ruohola-Baker et al., 1993). However, follicle cell clones ectopically expressing *rhomboid* have a nonautonomous effect on cell fate (Ward and Berg, 2005), consistent with the production of a diffusible molecule like Spi, and therefore differ markedly from *cic* mutant clones, suggesting that *rhomboid* is not a Cic target.

To address this issue, we generated *rhomboid* mutant follicle cell clones in a *cic* mutant background. If the *cic* mutant phenotype were mediated through ectopic *rhomboid*, these clones should suppress the associated cell fate changes. Visualization of BR-C expression revealed that *rhomboid* mutant clones have no effect on the ectopic appendage-producing fates in *cic* mutant egg chambers (Fig. 5F,F′), indicating that ectopic *rhomboid* expression is not the cause of the *cic* phenotype. Taken together, the failure of loss of *grk* or *rhomboid* to suppress the *cic* phenotype suggests that the ectopic induction of *mirr* and of appendage-producing fates in *cic* mutant ovaries is not a result of ectopic ligand-mediated Egfr activation.

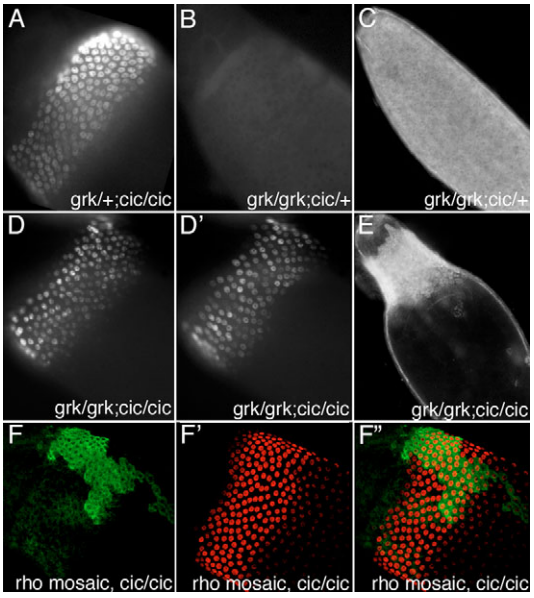

Fig. 5. Ectopic *mirr* expression in *cic* mutant egg chambers is *grk* independent. Anterior is to the upper left in all panels. (**A**) *cic* mutant egg chamber heterozygous for *grk* (lateral view, dorsal to the upper right) exhibiting ectopic *mirr-lacZ* expression. (**B**) grk^{2B6}/grk^{HK} egg chamber heterozygous for *cic*. The exposure time has been increased to emphasize the lack of *mirr-lacZ* expression. (**C**) Stage 14 grk^{2B6}/grk^{HK} egg chamber exhibiting a typical lack of dorsal appendages. (**D**,**D′**) Top and bottom focal planes of a grk^{2B6}/grk^{HK}; cic^{fetU6}/cic^{fetT6} egg chamber. Uniform *mirr-lacZ* expression is observed around the anterior circumference. (**E**) Stage 14 grk^{2B6}/grk^{HK}; cic^{fetU6}/cic^{fetT6} egg chamber. The eggshell lacks dorsal appendages but exhibits a pronounced collar of appendage material. (**F-F″**) cic^{fetU6}/cic^{fetT6} egg chamber with homozygous $rhomboid^{6}$ clones (ventral view). (F) $rhomboid^{6}$ clones (lack of green) including most of the follicular epithelium. (F′) The ectopic expression of high BR-C levels is unaffected. (F″) Merged image. Identical results were obtained with two different strong *rhomboid* alleles.

DISCUSSION

Cic represses appendage-producing follicle cell fate

In both homozygous mutant ovaries and mosaic follicular epithelia, loss of *cic* function results in the ectopic induction of molecular and morphological markers of appendage-producing fate, indicating that Cic regulates follicle cell fate determination. Taken together with the evidence that, in wild-type ovaries, Cic is downregulated in dorsal follicle cells during DV patterning (Goff et al., 2001), this observation suggests that downregulation of Cic by Egfr signaling establishes a dorsal domain of follicle cells competent to adopt an appendage-producing fate. This interpretation is consistent with the function of Cic in the developing wing, where Egfr-mediated downregulation of Cic is required for determination of wing vein fate (Roch et al., 2002). Cic is not required, however, to determine the fate of the dorsal midline follicle cells, which separate the appendage primordia, as this domain is established normally in the absence of *cic*.

Specification of appendage roof versus floor cell fates

High BR-C expressing cells make up the majority of the appendage primordium and form the roof of the appendage-producing epithelial tube, whereas the cells in a single row at the dorsal and anterior edges of the high BR-C domain form the tube floor (Dorman et al., 2004). The ectopic expression of high BR-C levels and apical constriction exhibited by *cic* mutant follicle cells are characteristic of the roof cell fate (Dorman et al., 2004), and the production by *cic* mutant clones of knob-like foci of appendage material, rather than elongated appendage-like structures, suggests that ectopic floor cell fates are not coordinately specified (Ward and Berg, 2005). However, ectopic appendage-like projections are observed at the

extreme anterior of eggs from *cic* mosaic egg chambers, suggesting that both roof and floor cells are determined in the more anterior clones, and cooperate to form an appendage-producing tube. This distinction may reflect the proximity of such clones to the source of Dpp, which is produced by the anterior-most follicle cells and specifies the AP position of the appendage primordia (Deng and Bownes, 1997; Dobens et al., 2000; Peri and Roth, 2000; Twombly et al., 1996), and therefore are could suggest a requirement for Dpp in floor cell fate determination.

Cic restricts appendage-producing fate through repression of *mirr*

We have shown that loss of *mirr* function suppresses the cell fate transformation caused by the loss of *cic*, providing evidence that *mirr* is a crucial regulatory target of Cic and is required for appendage-producing fate determination. We have also found that *mirr* is required to specify high BR-C expressing fates in the endogenous appendage primordia (M.R.A. and L.A.N., unpublished) and that ectopic expression of *mirr* in clones of follicle cells is sufficient to induce characteristics of this fate. These observations are consistent with previous evidence that decreased *mirr* function is correlated with reduced dorsal appendages and that ectopic *mirr* expression throughout the follicular epithelium can generate extra appendage material (Jordan et al., 2000; Zhao et al., 2000b). However, our direct analysis of follicle cell fate markers in clones with defined boundaries demonstrates that the expression of *mirr* is sufficient to induce both molecular and morphological markers of appendage-producing fate, and that this effect is cell autonomous. Such a role for *mirr* would be consistent with its function in other developmental contexts, where the localized expression of *Iro-C* genes defines cell identity (Cavodeassi et al., 2000; Cavodeassi et al., 2001; Cavodeassi et al., 2002; Dominguez and de Celis, 1998).

Cells engineered to express ectopic *mirr* have been reported to express ectopic *rhomboid* (Jordan et al., 2000; Schweitzer et al., 1995), which could in principle generate active Spi and lead to ectopic Egfr activity and changes in follicle cell fate. Our data show clearly that *mirr*-mediated induction of *rhomboid* is not the primary cause of the ectopic appendage-producing fates in *cic* mutant ovaries, as this phenotype is not suppressed by loss of *rhomboid* function. However, some level of *rhomboid* induction by ectopic *mirr* could lead to active Spi production, particularly near the endogenous *mirr* domain, which may in turn explain the rare cases of nonautonomy associated with *cic* mutant clones in this region.

cic mutant ovaries also fail to express *pipe* (*pip*), which is normally expressed in ventral follicle cells and defines the embryonic DV axis (Goff et al., 2001; Sen et al., 1998). Although neither *mirr* nor *fng* loss-of-function clones affect *pip* expression (Peri et al., 2002), ectopic overexpression of *mirr* has been reported to repress *pip* (Jordan et al., 2000). Multiple lines of evidence, however, indicate that ectopic expression of *mirr* in *cic* mutant ovaries does not account for their lack of *pip* expression. For example, ectopic *mirr* expression does not occur in *cic* mutant ovaries until stage 10B, well after the absence of *pip* is detected (Goff et al., 2001). In addition, loss of *cic* in ventral posterior follicle cells results in loss of *pip* without induction of ectopic *mirr* (see Fig. 3) (Goff et al., 2001). Taken together with the evidence that *rhomboid* is not required for the *cic* mutant phenotype (see above), these data indicate that Cic regulates *pip* independently of its effect on *mirr* expression.

Although our data demonstrate a role for *mirr* as an important determinant of appendage-producing fate, the relevant transcriptional targets of Mirr in this process remain to be identified. Although Mirr represses *fng* in dorsal anterior follicle cells (Jordan et al., 2000; Zhao et al., 2000a), the resulting *fng* boundary is not involved in DV patterning of appendage-producing fates. Recent microarray analysis has identified a number of genes that are regulated by *mirr* overexpression or alterations in Egfr signaling and that therefore represent potential Mirr targets (Jordan et al., 2005). Mirr also appears to regulate cell affinity, as *mirr* mutant clones in the eye exhibit a round shape (Yang et al., 1999). In dorsal anterior follicle cell patterning, this proposed function might contribute to the smooth boundary observed between the roof and floor cells of the appendage primordia (Ward and Berg, 2005), possibly contributing to appendage morphogenesis.

Cic blocks induction of *mirr* by anterior positional cues

In both *cic* homozygous egg chambers and *cic* mutant follicle cell clones, ectopic *mirr* expression is restricted to the anterior half of the epithelium, indicating that *mirr* is also regulated by positional information along the AP axis. Although in principle a posterior repressor could account for this effect, on the basis of prevailing models of follicular epithelium AP patterning (see below), we favor the hypothesis that expression of *mirr* requires positive input from an anterior positional cue. We propose that, in wild-type ovaries, Cic blocks the induction of *mirr* by this anterior signal. However on the dorsal side, where Cic becomes downregulated, this signal is not blocked, leading to the induction of *mirr* expression and appendage-producing fate. In *cic* mutant ovaries, the anterior signal induces *mirr* expression throughout the DV axis.

A likely candidate for an anterior signaling molecule required for *mirr* expression is Dpp, which is produced by the anterior-most follicle cells and regulates gene expression along the AP axis (Deng and Bownes, 1997; Dobens et al., 2000; Peri and Roth, 2000; Twombly et al., 1996). Coordinate regulation of *mirr* along the DV and AP axes provides a molecular explanation for the observation that appendage-producing fates are determined at the intersection of Egfr and Dpp signaling (Deng and Bownes, 1997; Peri and Roth, 2000). Regulation of *mirr* by an anterior cue such as Dpp could also explain the observation that *mirr* expression in *cic* mutant ovaries is normal until stage 10B; although the *cic* mutant cells are competent to express *mirr*, detectable levels may not be induced until the posterior migration of anterior follicle cells in mid-oogenesis brings the source of Dpp to the anterior margin of the oocyte (Guichet et al., 2001).

In addition to invoking an anterior signal in the regulation of *mirr*, our data indicate that *mirr* is also positively regulated by dorsally restricted Egfr signaling, independent of Cic. *cic* mutant egg chambers exhibit ectopic *mirr* throughout their anterior circumference, but *mirr* levels remain highest dorsally. In *grk;cic* double mutant egg chambers this dorsal high point of *mirr* expression is abolished, suggesting that the wild-type dorsal anterior *mirr* expression pattern is the result of both dorsal and anterior inputs.

Model for patterning of the follicular epithelium along the DV axis

Collectively, our data support a model in which Cic blocks the induction of *mirr* expression and appendage-producing fates in response to an anterior signal, for example Dpp. Egfr-mediated downregulation of Cic in dorsal anterior follicle cells therefore allows these cells to respond to Dpp, contributing to the dorsal anterior *mirr* expression pattern (Fig. 6A, top), whereas the presence of Cic in ventral and lateral follicle cells blocks their

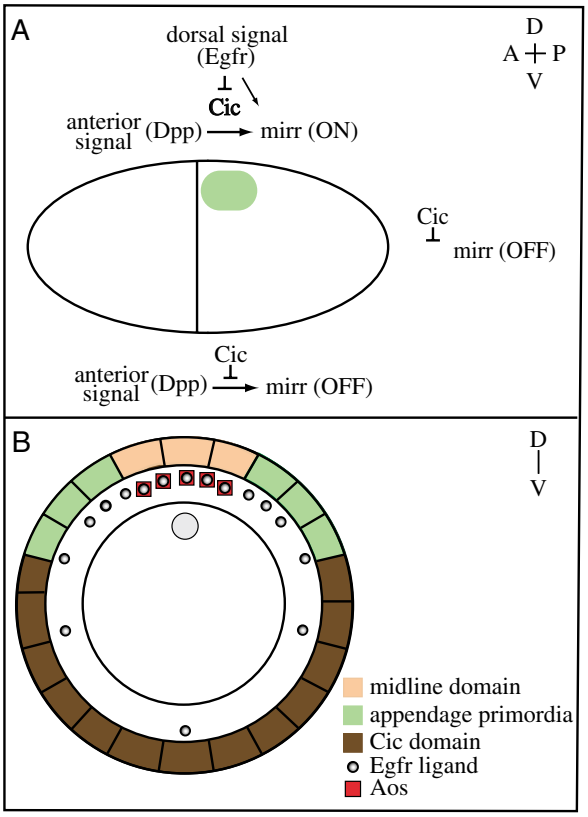

Fig. 6. Model for integration of AP and DV positional information and eggshell patterning. (**A**) Cartoon depicting a lateral surface view of a stage 10B egg chamber. A single appendage primordium (green) is shown. Cic is downregulated in dorsal follicle cells, allowing Dpp to induce *mirr* expression; dorsally-localized Egfr activation also positively regulates *mirr* independently of Cic. Cic remains present in the remaining follicle cells and prevents the induction of *mirr* by an anterior positional cue, probably Dpp. (**B**) Diagram of an anterior cross section through an egg chamber, illustrating the follicular epithelium (shaded outer section) surrounding the oocyte (inner large circle). The oocyte nucleus (gray circle in oocyte) marks the dorsal side. Cic (dark brown) is lost from dorsal follicle cells where Egfr signaling exceeds a certain threshold, rendering these follicle cells competent to express *mirr* and adopt an appendage-producing fate (green), and thus defining the ventral limit of the appendage primordia. As proposed previously (Wasserman and Freeman, 1998), Aos (red) is induced in dorsalmost follicle cells in response to higher levels of Egfr activation (circles), resulting in local downregulation of Egfr activation and generating a dorsal midline fate (light brown) that marks the dorsal limit of the appendage primordia.

response to this cue (Fig. 6A, bottom). Within the dorsal Cic-free domain, the Rhomboid/Spi/Aos autocrine-feedback loop would regulate Egfr activity to resolve two distinct appendage primordia (Fig. 6B) (Peri et al., 1999; Wasserman and Freeman, 1998). In *cic* mutant egg chambers, all follicle cells would be competent to respond to the anterior signal, resulting in ectopic *mirr* expression and appendage-producing fate in the anterior follicle cells that receive the signal.

Previous work has shown that the appendage primordia are determined at the intersection of dorsal and anterior signals (Deng and Bownes, 1997; Peri and Roth, 2000), and the simplest interpretation has been that these signals function additively to specify appendage-producing fate. Instead, however, the demonstration that the distribution of Cic along the DV axis determines the competence of follicle cells to respond to AP patterning signals reveals unexpected crosstalk between DV and AP patterning signals, and indicates that Cic integrates these pathways.

Along the DV axis, we propose that the pattern of the follicular epithelium is determined by the function of two Egfr targets, Cic and Aos, in distinct domains (Fig. 6B). High levels of Egfr activity induce production of Aos at the dorsal midline, where it antagonizes Spi, thus splitting the initial dorsal domain of Egfr activity and defining the dorsal limits of the appendage primordia. Lower levels of Egfr signaling are sufficient to downregulate Cic, defining a dorsal domain that lacks Cic and is therefore competent to adopt dorsal fates. Cic remains present in ventral and lateral follicle cells, where it blocks the induction of crucial transcriptional targets, such as *mirr*, by Dpp. The dorsal limit of the Cic domain thus defines the ventral limit of the appendage primordia. Cic-mediated repression of target genes may represent a general mechanism for the integration of multiple spatial inputs in a developing tissue.

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