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Drosophila bunched integrates opposing DPP and EGF signals to set the operculum boundary

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SUMMARY

The *Drosophila* BMP homolog DPP can function as a morphogen, inducing multiple cell fates across a developmental field. However, it is unknown how graded levels of extracellular DPP are interpreted to organize a sharp boundary between different fates. Here we show that opposing DPP and EGF signals set the boundary for an ovarian follicle cell fate. First, DPP regulates gene expression in the follicle cells that will create the operculum of the eggshell. DPP induces expression of the enhancer trap reporter A359 and represses expression of *bunched*, which encodes a protein similar to the mammalian transcription factor TSC-22. Second, DPP signaling indirectly regulates A359 expression in these cells by downregulating expression of *bunched*. Reduced *bunched* function restores A359 expression in cells that lack the Smad protein MAD; ectopic expression of BUNCHED suppresses A359 expression in this region. Importantly, reduction of *bunched* function leads to an expansion of the operculum and loss of the collar at its boundary. Third, EGF signaling upregulates expression of *bunched*. We previously demonstrated that the *bunched* expression pattern requires the EGF receptor ligand GURKEN. Here we show that activated EGF receptor is sufficient to induce ectopic *bunched* expression. Thus, the balance of DPP and EGF signals sets the boundary of *bunched* expression. We propose that the juxtaposition of cells with high and low BUNCHED activity organizes a sharp boundary for the operculum fate.

Key words: Oogenesis, *bunched*, TSC-22, *decapentaplegic*, TGFβ family signaling

INTRODUCTION

The *Drosophila* TGFβ family member DECAPENTAPLEGIC (DPP) (Padgett et al., 1987) is a potent inducer of developmental fate (Raftery and Sutherland, 1999). In some tissues, DPP functions as a morphogen, inducing multiple fates in a concentration-dependent fashion (reviewed in Neumann and Cohen, 1997). In other tissues, such as the longitudinal wing veins, DPP acts locally to specify a distinct cell fate with a sharp boundary (de Celis, 1997). In either situation, it is not known how the boundary to a DPP-induced fate is organized. Here we show that DPP patterns a domain of the ovarian follicle cells that will give rise to the operculum of the eggshell. We further show that the *bunched* (*bun*) gene (Dobens et al., 1997; Kania et al., 1995), also known as *shortsighted* (Treisman et al., 1995), is required both to limit the size of the operculum and to form the collar at the operculum boundary. Enhancer trap reporters in *bunched* are negatively regulated by DPP and positively regulated by EGF receptor (EGF-R), indicating that *bunched* integrates opposing DPP and EGF signals to set the boundary to a DPP-induced fate.

bunched encodes a protein similar to TSC-22, a TGFβ early response gene in mouse osteoblasts (Dobens et al., 1997; Shibanuma et al., 1992; Treisman et al., 1995). TSC-22 family members have a leucine zipper similar to bZIP transcription factors (Hamil and Hall, 1994; Shibanuma et al., 1992). In lieu of the basic domain of bZIP proteins, members of the TSC-22 gene family share a conserved sequence called the TSC domain (Dobens et al., 1997), which may function as a DNA-binding domain (Ohta et al., 1996; Seidel et al., 1997). Human TSC-22 can bind an essential region of the C-type natriuretic peptide gene promoter (Ohta et al., 1996), suggesting that TSC-22-like proteins directly regulate gene expression.

Initial studies implicated *bunched* in both DPP and EGF genetic pathways. Analysis of *bunched* function in eye development revealed genetic interactions with *dpp*, as well as with *anterior open*, which encodes a transcription factor regulated by receptor tyrosine kinases (Treisman et al., 1995). However, *bunched* mutant phenotypes in the eye (Treisman et al., 1995) and in the embryo (Kania et al., 1995; Perrimon et al., 1996) did not reveal specific roles for the gene. During oogenesis, *bunched* is a target gene for EGF signaling (Dobens et al., 1997). *bunched* shows patterned expression in the follicle cells (FC) (Dobens et al., 1997), which surround the

developing oocyte and secrete the eggshell (reviewed by Spradling, 1993). *bunched* expression in the FC requires *gurken (grk*) (Dobens et al., 1997), a TGF-α-like gene that specifies the dorsal-ventral axis of the egg chamber (Neuman-Silberberg and Schüpbach, 1993; Schüpbach, 1987). Consistent with this, *bunched* is required in dorsal appendage formation (Dobens et al., 1997). The follicle cells are an excellent system to examine the role of *bunched* in interactions between DPP and EGF.

EGF signaling induces dorsal FC fates, which include the operculum and dorsal appendage structures of the eggshell (reviewed in Perrimon and Perkins, 1997; Ray and Schüpbach, 1996). The ligand GRK is secreted by the oocyte (Neuman-Silberberg and Schüpbach, 1993) and acts through the EGF-R in the FC (Price et al., 1989; Schejter and Shilo, 1989; Schüpbach, 1987). GRK patterns the dorsal-anterior FC during stage 10 of oogenesis, when these FC overlie the oocyte (Ray and Schüpbach, 1996; Spradling, 1993). Subsequently, additional EGF ligands are expressed or activated in the dorsal-anterior FC, and contribute to formation of the dorsal appendages (Sapir et al., 1998; Wasserman and Freeman, 1998). However, it is not known how the dorsal anterior domain is subdivided along the anterior-posterior axis to create the operculum and dorsal appendage fates.

Recently, it has been suggested that DPP patterns the anterior FC (Deng and Bownes, 1997; Twombly et al., 1996). The size and placement of the operculum and dorsal appendages are quite sensitive to altered levels of DPP signal (Twombly et al., 1996). DPP is expressed in anterior nurse cell-associated FC from stage 8 onward, although these cells do not contribute to the final eggshell. The operculum is formed by anterior dorsal columnar FC after they migrate centripetally to enclose the anterior end of the oocyte (Spradling, 1993).

Here we define an anterior domain of columnar FC that directly responds to DPP just before centripetal migration begins. Reduced *bunched* function causes an expansion of this domain and of the operculum. Thus, *bunched* is required to limit the operculum fate. We further demonstrate that DPP activates operculum gene expression indirectly by inactivating *bunched*. The pattern of *bunched* expression is due to repression by DPP and activation by EGF receptor; *bunched* expression forms a boundary at the centripetal migrating FC. We conclude that *bunched* expression is an integrated readout of both DPP and GRK signals, and establishes the boundary to a DPP-induced fate.

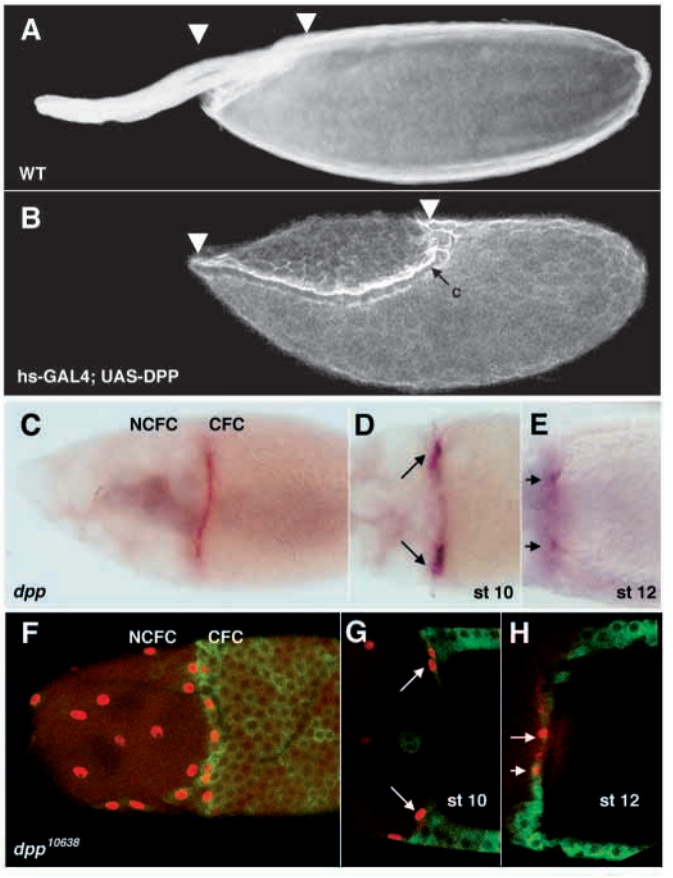

Fig. 1. Anterior eggshell structures are sensitive to DPP expressed in the nurse cell FC. Anterior is left and dorsal up in all panels. (A) Wild-type eggshell, with dorsal appendages. The approximate size of the operculum is defined by the arrowheads. (B) Expansion of operculum in eggs laid by females genetically *hs-GAL4; UAS-dpp*. After 1 hour heat shock and 24 hour incubation at 29°C, eggs laid have an expanded operculum (between arrowheads). The position of the operculum, cell imprints and collar (c) are normal. (C-H) *dpp* transcripts and reporter gene expression are localized to the NCFC and leading edge centripetal migrating FC. (C) Surface view shows *dpp* mRNA in a set of NCFC at the border of the columnar FC (CFC); *dpp* transcripts in the stretched NCFC are difficult to see in this plane of focus. (D,E) Optical cross-sections showing strong accumulation of *dpp* transcripts in cells at the leading edge of centripetal migration (LEFC). (F-H) DPP expression in the nurse cell FC revealed by *lacZ* expression (red) from enhancer trap dpp^{10638} (Twombly et al., 1996). All follicle cells express hs-N-MYC (green). (F) Surface view showing DPP expression in the nurse cell FC. (G) Cross-section showing the nurse cell/oocyte boundary of a stage 10 egg chamber. dpp^{10638} is expressed in the LEFC (arrows). (H) Cross-section of stage 12 egg chamber showing dpp^{10638} expression in set of cells in the LEFC (arrows).

MATERIALS AND METHODS

Drosophila strains

All genes are described in Flybase (The FlyBase Consortium, 1999). bun^{4230} and bun^{r143} were previously described as strong and weak alleles of *shs*, respectively (Treisman et al., 1995). All *bunched* mutations are due to insertion of a P element carrying a *lacZ* reporter; only bun^{r143} does not express β-galactosidase in the ovary. The enhancer trap line A359 (*l(3)A359.1M3*) has been described (Dobens and Raftery, 1998; Grossniklaus et al., 1989). Transgenic strains used were: UAS-*λtop1* (Queenan et al., 1997); *actin>CD2>GAL4* (Pignoni and Zipursky, 1997); *Tub1>*f^{+}*>dpp* (Zecca et al., 1995); *hs-FLP1* (Golic, 1991); *hs-FLP2* (from Allan Spradling); *Sb hs-FLP, hs-N-MYC* and *FRT40A* (Xu and Rubin, 1993); *pUmMad* (Sekelsky et al., 1995); *hs-GAL4* (from Norbert Perrimon); *UAS-dpp* (Staehling-Hampton and Hoffmann, 1994).

UAS-BUN constructs

The UAS-BUN-1 and UAS-BUN-2 constructs were made by cloning the full-length BUN-1 and BUN-2 cDNAs into the *Eco*RI site of pUAST (Brand and Perrimon, 1993). BUN-X was generated by site-directed mutagenesis of the BUN-1 cDNA, using the Sculptor in vitro mutagenesis kit (Amersham) and the oligonucleotide

GACCAGATCCATTGGCTGCACGATTTCGTTATCAATCGC. The amino acid sequence VAIDNKIEQAMD (aa 54-65) was changed to VAIDNEIVQPMD, confirmed by DNA sequencing. An *Eco*RI fragment containing the full-length mutant cDNA was cloned into pUAST. Transgenic lines were generated as described (Rubin and Spradling, 1982).

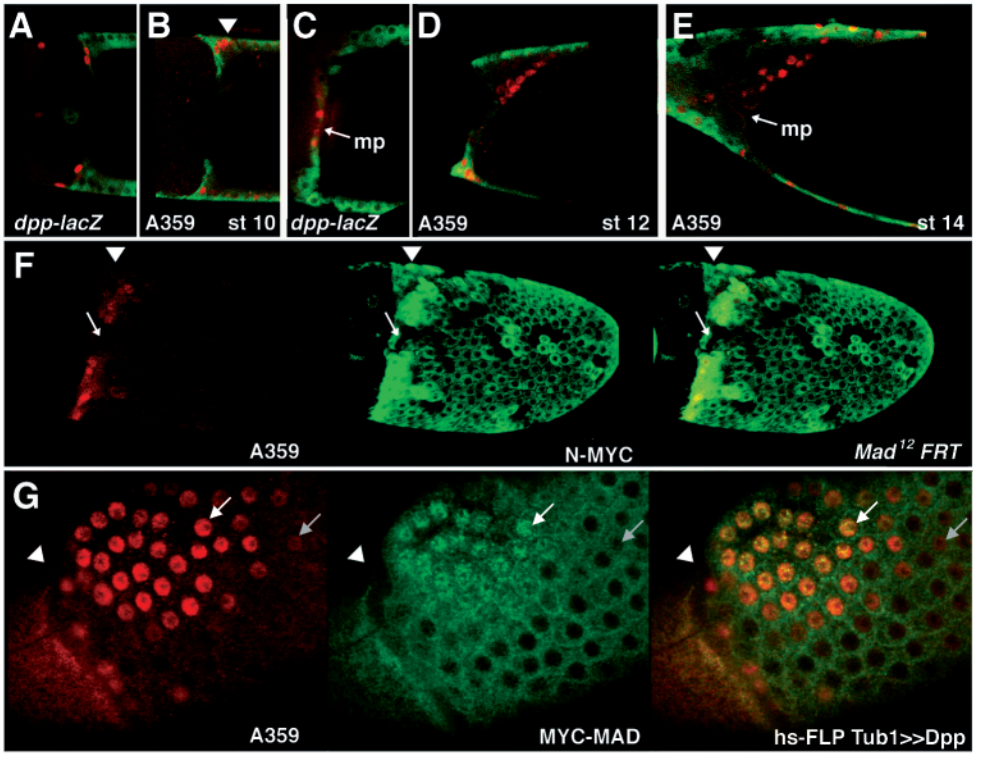

Fig. 2. DPP is necessary and sufficient for operculum gene marker A359 expression. For panels A-E, all follicle cells express the hs-N-MYC marker (green); cells expressing nuclear-β-galactosidase are detected by rhodamine staining (red). (A) *dpp*^{10638} expression (red) in the NCFC and LEFC at stage 10. (B) At stage 10, A359 expression (red) marks a 1- to 2-cell-wide strip of anterior columnar FC adjacent to, but not overlapping, DPP-expressing cells. (C) *dpp-lacZ*-expressing cells surround the micropyle (mp) at stage 12. (D) A359 is expressed in most cells that have migrated to cover the anterior oocyte, but is not expressed in the cells that surround the micropyle at center. (E) A359 expression persists in the dorsal operculum-forming FC and ventral collar-forming cells. At stage 14, lower levels can be detected in the dorsal appendage-forming FC and the FC stretched over the oocyte. (F) Double immunofluorescently stained egg chamber with homozygous null *Mad* clones (genotype *hsFLP1; Mad*^{12} *FRT 40A/hs-N-MYC FRT 40A*). β-galactosidase from A359 is detected with rhodamine. Clones are marked by the loss of the N-MYC epitope, detected with fluorescein. Right panel: combined image shows that anterior A359 expression does not occur in centripetal migrating FC (arrowhead) that are homozygous for *Mad*^{12} (arrow). Middle panel: fluorescein staining alone, showing the absence of N-MYC antigen in four *Mad*^{12} cells in the anterior columnar FC. Left panel: rhodamine staining alone, showing the difference in levels of A359 expression between wildtype and *Mad* null cells (arrow) within the centripetal migrating FC. (G) Double immunofluorescent staining for MYC-MAD (center panel), β-galactosidase expression from A359 (left panel) and a merged image (right panel) following induction of small clones of follicle cells that constitutively express *dpp* from the tubulin promoter (genotype *hsFLP1; Tub>f*$^{+}$*>dpp; A359*). Ectopic A359 expression is coincident with cells that show MYC-MAD in the nucleus. Cells that do not express A359 do not have detectable levels of MYC-MAD in the nucleus. In cells that express low levels of A359, MYC-MAD is detected in the nucleus, but at a lower level than in the cytoplasm (gray arrow). In cells that express high levels of A359, MYC-MAD is either concentrated in the nucleus or distributed relatively evenly between the cytoplasm and the nucleus (white arrow). The small, non-staining spot in each nucleus is the nucleolus. Arrowheads mark the edge of the normal A359 expression domain (B,F,G).

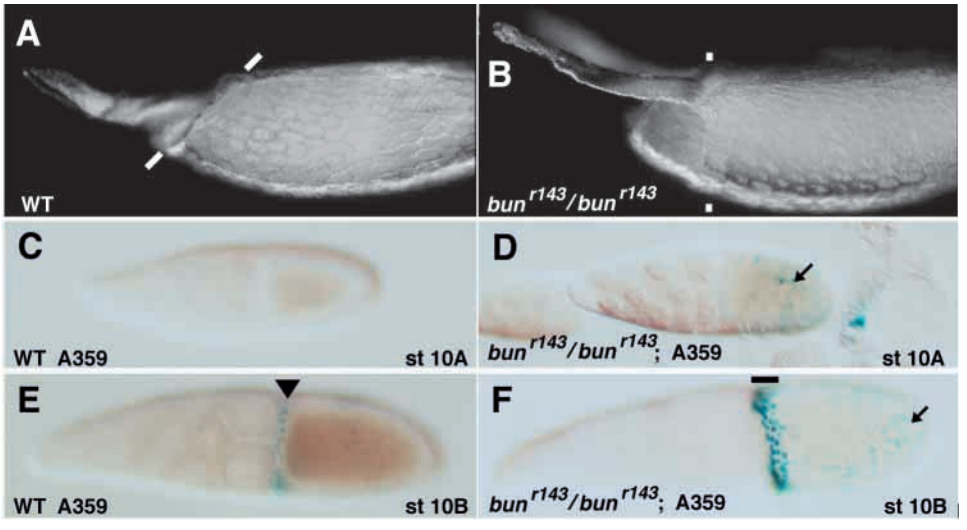

Fig. 3. *bunched* is necessary to limit the operculum fate. In all panels, anterior is left. (A) Wild-type eggshell with focus on the operculum collar. The normal collar forms at an angle (flanking white bars). (B) Eggs laid by a *bun*^{r143} homozygous females have defective eggshells: the operculum is slightly expanded and operculum cell imprints extend ventrally; the collar is absent and the boundary between operculum imprints and mainbody imprints is rotated ventrally (flanking white bars). (C-F) Expression of A359 is expanded in *bun*^{r143} homozygous females. (C,E) Expression of A359 is normal in *bun*^{r143}/+ females: absent at stage 10A (C) with onset of detectable expression at stage 10B (E, arrowhead). (D,F) Stage 10A egg chambers from *bun*^{r143}/*bun*^{r143} females show precocious expression of A359 in posterior FC (arrow) (D). By stage 10B, posterior expression of A359 is elevated (arrow) and anterior expression is stronger and covers a broader domain (bar) (F).

Generation of homozgyous mutant clones

Homozygous clones were created using heat-shock-induced, FLP/FRT-mediated recombination and visualized by loss of the N-MYC epitope as described (Xu and Rubin, 1993). *Mad*^{12} *FRT40A*, *Mad*^{12} *bun*^{r143} *FRT40A*, or *Mad*^{12} *bun*^{4230} *FRT40A* was used as indicated. In both these and the flip-out clone experiments, clones were rarely recovered in the anteriormost columnar follicle cells. Females were heat-shocked for 1 hour at 37°C; ovaries were dissected 3 days later.

Generation of flip-out clones

Clones of cells expressing DPP from the *Tubα1* promoter were generated using the genotype *hs-FLP; Tub>f*$^{+}$*>dpp/pUmMad; A359*. Clones of cells expressing GAL4 from the Actin5C promoter were generated in the genotypes: (1) *hsFLP1/actin5C>CD2>GAL4; UAS-BUN-1/UAS-GFP A359.1M3*, (2) *UAS-BUN-X/actin5C>CD2> GAL4; hs-FLP2; UAS-GFP A359.1M3*, (3) *hsFLP1/actin5C>CD2> GAL4; UAS-dpp/bun*^{4230}*; UAS-GFP* or (4) *UAS-λtop1/actin5C> CD2>GAL4; bun*^{4230}*; UAS-GFP/Sb hs-FLP*. Females were heat shocked for 30-60 minutes at 37°C; ovaries were dissected 2 days later.

Histochemical and immunofluorescence staining

Dissected ovaries were fixed in 4% paraformaldehyde (Polysciences, Inc.) in PBS for 5-15 minutes. β-galactosidase activity was detected as described (Simon et al., 1985). For immunofluorescence, disrupted ovaries were washed in PSN (Xu and Rubin, 1993) and incubated overnight in mouse anti-MYC (1:200 dilution; Oncogene Science) and rabbit anti-β-galactosidase (1:10,000, pre-adsorbed to fixed tissue; Cappel). Secondary antibodies were from Jackson Labs. Images were collected using a Leica TCS-NT confocal microscope and figures were prepared using Photoshop.

In situ hybridization

Using the small *bunched* cDNA, a large cDNA was isolated from an ovarian library (Stroumbakis et al., 1994). This cDNA is the same size as the large cDNA isolated from eye disks (Treisman et al., 1995), suggesting that it encodes BUN-2. Antisense RNAs specific to each isoform were produced from subcloned regions: nucleotides 2960-3938 for the large cDNA and nucleotides 0-962 for the small cDNA. Whole-mount in situ hybridization was performed as described (Wasserman and Freeman, 1998).

RESULTS

Anterior eggshell structures are sensitive to DPP levels

DPP is expressed in the anterior follicle cells and perturbed DPP signaling leads to anterior eggshell defects (Twombly et al., 1996). In particular, two dorsal anterior structures, the operculum and the dorsal appendages (Fig. 1A), are sensitive to altered DPP levels (Twombly et al., 1996). Here, we illustrate the effects of a global increase in DPP levels, using heat-shock-GAL4 to drive UAS-*dpp* expression throughout the FC (Fig. 1B). The resultant eggs showed expanded opercula and reduced dorsal appendages (Fig. 1A,B). In other respects, the eggshells were normal. At the extreme anterior, normal micropyles were formed. The mutant opercula generally had a normal organization of large cell imprints surrounded by a raised structure, the collar (c, Fig. 1B). Significantly, expansion of the operculum always occurred over the dorsal side of the egg, indicating that dorsal-ventral patterning was unperturbed. A simple hypothesis, consistent with DPP's role in axial patterning of other tissues, is that DPP patterns the anterior FC (Twombly et al., 1996).

Dorsal-ventral patterning of the follicle cells occurs during stage 10 of oogenesis, when the anterior columnar FC overlay the oocyte nucleus. Thus, we expected that DPP-dependent patterning of anterior FC occurred at about the same stage. We examined DPP expression using both in situ hybridization to *dpp* transcripts and immunofluorescence confocal microscopy to detect β-galactosidase from the *dpp-lacZ* enhancer trap, *dpp*^{10638}. At the onset of stage 10, DPP is expressed in the nurse cell FC (NCFC, Fig. 1C-H). Slightly later, during stages 10B and 11, *dpp* transcripts accumulate in FC at the leading edge of centripetal migration (Fig. 1D,E). At the end of migration, a few *dpp*-expressing cells surround the micropyle at the anterior edge of the forming operculum (data not shown, and Twombly et al., 1996). The *dpp* enhancer trap faithfully reproduces this pattern of expression (Fig. 1F-H). These observations are consistent with a model in which a gradient of DPP defines the number of cells recruited to the operculum fate.

A359 enhancer trap expression defines a DPP-responsive domain

To obtain early markers for follicle cell fates specified by DPP, we sought *lacZ* enhancer trap insertions expressed adjacent to the DPP-expressing cells. *l(3)A359.1M3* (Grossniklaus et al., 1989), hereafter called A359, is expressed in an anterior band of columnar FC (Fig. 2B-D), directly adjacent to, but not overlapping, the DPP-expressing cells (Fig. 2A,B and data not shown). Shortly after the onset of A359 expression, both *dpp*- and A359-expressing cells migrate centripetally to cover the anterior portion of the oocyte (Figs 1E,F and 2B,C, respectively). At later stages, high levels of A359 expression persist in the centripetal migrating FC while basal levels were detected in other FC groups (Fig. 1D).

To determine whether DPP signaling is necessary for high-level A359 expression, we generated clones of follicle cells unable to respond to DPP. *Mad* encodes a Smad protein essential for DPP signal transduction (reviewed in Raftery and Sutherland, 1999), so we generated clones homozygous for the null allele *Mad*^{12} (Xu and Rubin, 1993). Numerous, small *Mad*^{12} clones were recovered throughout the columnar FC, identified by the absence of the N-MYC marker. Five clones fell within the domain where A359 is normally expressed; in these clones, A359 was expressed only at basal levels (Fig. 2F). Thus, DPP signal transduction through MAD is necessary for the high level of A359 expression in anterior columnar FC.

If DPP patterns anterior FC fates, it should be sufficient to induce A359 expression. We found that ectopic A359 expression was induced by DPP overexpression in stage 10B or later egg chambers, when DPP was expressed transiently with a heat-shock promoter (Dobens and Raftery, 1998). Constitutive expression of DPP from the tubulin promoter in flip-out clones (Zecca et al., 1995) led to disruptions of the FC epithelium by stage 10B; in some cases, ectopic A359 expression was present (data not shown, and Fig. 2G).

To directly compare A359 expression with DPP responses, we examined nuclear MAD using a transgenic MYC-tagged MAD (MYC-MAD) construct that rescues *Mad* mutants (Sekelsky et al., 1995). Consistent with studies of other tissues (Newfeld et al., 1997), nuclear MYC-MAD was undetectable

in wild-type egg chambers. However, heat-shock-induced DPP expression reliably caused detectable, but transient, nuclear localization of MYC-MAD at stage 10. In contrast, constitutive DPP expression from flip-out clones was rarely associated with detectable nuclear MYC-MAD by stage 10. One stage 10B egg chamber was recovered that showed ectopic MYC-MAD nuclear localization; all cells with MYC-MAD detectable in the nucleus showed ectopic expression of A359 (Fig. 2G). In cells that expressed low levels of A359, low levels of MYC-MAD were present in the nucleus (gray arrow, 2G); whereas cells with high levels of A359 had higher nuclear levels of MYC-MAD (white arrow, 2G). In sum, DPP signaling through MAD is both necessary and sufficient to induce A359 expression.

bunched sets the posterior limit of the operculum fate

Previously, we reported that *bunched* is required in the dorsal anterior follicle cells for proper dorsal appendage formation (Dobens et al., 1997). During the course of clonal analyses, we noted that induction of *bunched* mutant clones was associated with increased fragility of the FC epithelium and the resultant eggshell (data not shown). Here we demonstrate that *bunched* is required in the columnar FC to repress the operculum fate.

To further investigate the requirements for *bunched*, we examined eggs laid by females homozygous for a weak *bunched* allele, bun^{r143} (Treisman et al., 1995). These females are sterile, both due to low fecundity and to maternal effect lethality. The eggs had minor defects in the dorsal appendages (94%, *n*=96; Fig. 3B), consistent with previous results (Dobens et al., 1997). The most striking defects were altered opercula (85%; Fig. 3B), which were expanded posteriorly and ventrally. Every eggshell with an enlarged operculum also was missing the collar structure at the operculum edge. These phenotypes indicate that *bunched* limits the size of the operculum and is required to form the collar at the operculum boundary.

To determine the origin of the eggshell defects, we examined the expression of A359 in egg chambers from bun^{r143} homozygous females. In wild-type females, A359 expression was absent at stage 10A (Fig. 3C). Normal A359 expression began during stage 10B, just prior to centripetal migration (Fig. 3E). In contrast, bun^{r143} homozygous FC exhibited precocious A359 expression before stage 10A (Fig. 3D). By stage 10B, mutant egg chambers showed an expanded domain of strong A359 expression (Fig. 3F). Thus, decreased *bunched* function resulted in expansion of both the operculum of the eggshell and A359 enhancer trap expression in the FC. We conclude that *bunched* acts before or during stage 10 to repress operculum formation.

The sensitivity of the ventral operculum boundary to reduced *bunched* function was a surprise. We previously reported that *bunched* mRNA accumulates at high levels in dorsal anterior FC, with low levels throughout the egg chamber (Dobens et al., 1997). β-galactosidase activity staining revealed only the dorsal-anterior expression from several enhancer traps in the gene (Dobens et al., 1997). However, sensitive immunofluorescence staining for β-galactosidase protein indicated a low level of expression in most of the FC (Fig. 4B). The only populations that lacked detectable expression were the anterior band of columnar FC at stage 10, and the centripetal migrating FC at later stages. These populations include the cells that will make the operculum.

To more precisely map *bunched* expression, we examined the expression of each of the two *bunched* mRNAs. The previously reported expression pattern is a composite for both transcripts (Dobens et al., 1997). Alternative 5′ exons give rise to a small and a large transcript, which encode two protein isoforms (Treisman et al., 1995). Both isoforms contain the leucine zipper and TSC domain (highlighted in Fig. 5B) and the small isoform, BUN-1, is similar in size to vertebrate TSC-22. A short probe specific to each transcript was used for in situ hybridization to ovaries (Wasserman and Freeman, 1998). Each transcript was strongly expressed in the nurse cells from stages 8-12, consistent with the maternal effect phenotype (Perrimon et al., 1996).

Expression in the follicle cells was first detected at stage 8 (Fig. 4C). The BUN-1 transcript showed a pattern of expression similar to the pattern of the enhancer trap reporters (Fig. 4B versus D). At stage 10, expression was highest in dorsal FC, but also present in ventral FC (Fig. 4D). Strong nurse cell expression obscured the centripetal migrating FC at this stage. However, by stage 12, it could be seen that BUN-1 expression was strongly reduced in the operculum-forming FC (Fig. 4E,F). The BUN-2 transcript showed a more restricted pattern, with strong expression in the dorsal anterior FC beginning at stage 10, and expression in the operculum-forming FC at stage 12 (data not shown). The combined high level expression of both *bunched* transcripts in the dorsal anterior FC may explain why the operculum boundary is most sensitive to reduced *bunched* function on the ventral side. The BUN-1 expression pattern is consistent with a role in repressing the operculum fate.

DPP inactivates BUNCHED to indirectly promote the operculum fate

Given that *bunched* opposes A359 expression and operculum formation, we wished to determine whether DPP indirectly induces the operculum fate by inactivating *bunched*. If DPP acts indirectly through *bunched*, then cells mutant for both *Mad* and *bunched* should express A359. Conversely, if DPP signaling directly induces A359 expression, then cells that lack both *Mad* and *bunched* should not express A359. For this experiment, we used the weak allele bun^{r143}, because it is the only allele that does not express *lacZ* in the FC. Anterior columnar FC homozygous for both Mad^{12} and bun^{r143} showed normal A359 expression (*n*=21; Fig. 5A). Thus, DPP induces A359 expression by inactivating *bunched*.

To determine whether BUNCHED is sufficient to block A359 expression in the anterior FC, we ectopically expressed each BUNCHED isoform. Random clones were induced using a flip-out GAL4 transgene (Pignoni and Zipursky, 1997) for the GAL4/UAS expression system (Brand and Perrimon, 1993). Ectopic expression of the large BUN-2 isoform had no effect on A359 expression (*n*=13; data not shown). In contrast, ectopic expression of BUN-1 was sufficient to block A359 expression in a cell autonomous manner (*n*=6; Fig. 5C) and led to disruptions in centripetal migration (data not shown). These data indicate that BUN-1 acts downstream of the DPP signal transducer MAD to repress A359 expression.

Overexpression of a mutant form of BUN-1 lacking the TSC domain (BUN-X) was not sufficient to block A359 expression

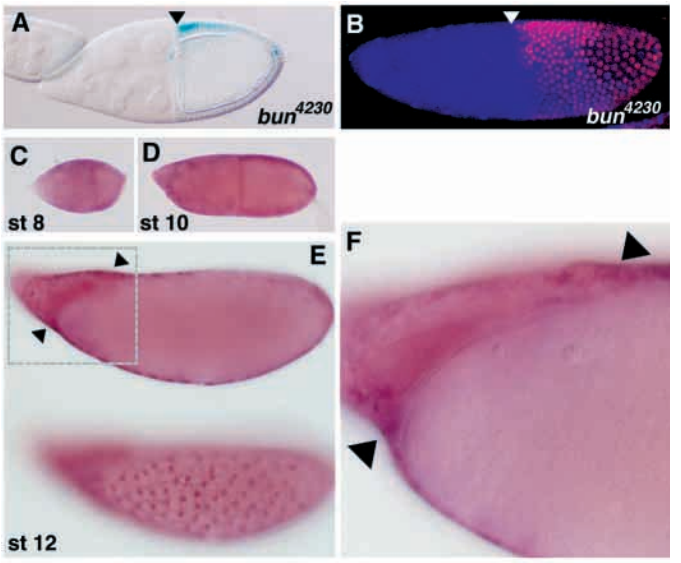

Fig. 4. Accumulation of BUN-1 mRNA parallels *bunched-lacZ* enhancer trap expression. (A) Activity staining for β-galactosidase expressed from *bun*^{4230} shows expression in the dorsal anterior FC but not in the centripetally migrating FC (CMFC, arrowhead). (B) Immunostaining of *bun*^{4230} for β-galactosidase (red) reveals wider expression, strong in the dorsal anterior FC, but detectable in posterior and ventral FC and in the NCFC (all nuclei are blue). Levels in the CMFC are undetectable. (C) BUN-1 mRNA is detected at stage 9 throughout the follicle cells and in the nurse cells and oocyte; (D) at stage 10, BUN-1 is slightly stronger in the dorsal columnar FC. (E) At stage 12 BUN-1 is in most FC subgroups except the CMFC (arrowhead) (F) 2× view of area outlined in gray (E).

(*n*=10; Fig. 5D). We are confident that BUN-X was expressed, because FC that expressed BUN-X were aberrant at later stages of oogenesis. Thus BUNCHED repression of A359 requires an intact TSC domain, consistent with the proposed function of this domain in DNA binding (Ohta et al., 1996; Seidel et al., 1997).

The balance of DPP and EGF signals defines the *bunched-lacZ* expression pattern

We have shown that genetic reduction of *bunched* function bypasses the requirement for MAD in anterior A359 expression and that BUN-1 transcript is not detected in the operculum-forming FC. A simple model to account for these data is that DPP promotes the operculum fate by preventing expression of BUN-1. To test this model, we asked whether *bunched-lacZ* is expressed in clones homozygous for *Mad*^{12}. In this experiment, *Mad*^{12} homozygous clones were also homozygous for *bun*^{4230}, the *bunched-lacZ* insertion mutation, because the *Mad* and *bunched* genes are on the same chromosome arm. In a separate experiment, FC homozygous for the *bun*^{4230} mutation alone showed normal *bunched-lacZ* expression levels (data not shown). In the posterior, *Mad*^{12} clones showed normal *bunched-lacZ* expression (Fig. 6E). In contrast, mutant clones in the anterior columnar FC showed ectopic expression of *bunched-lacZ* (*n*=8; Fig. 6E). The DPP type I receptor TKV is also required to prevent *bunched-lacZ* expression in this domain (data not shown). Thus, DPP signaling is necessary to prevent *bunched* expression in the anterior follicle cells.

To determine if DPP is sufficient to prevent *bunched-lacZ* expression, we first examined the effect of random small clones of cells that constitutively express *dpp*. We used a flip-out GAL4 driver (Pignoni and Zipursky, 1997) so that cells expressing UAS-DPP were autonomously marked with UAS-GFP. Ectopic DPP was associated with ectopic repression of *bunched-lacZ* (*n*=15). However, we also observed persistent

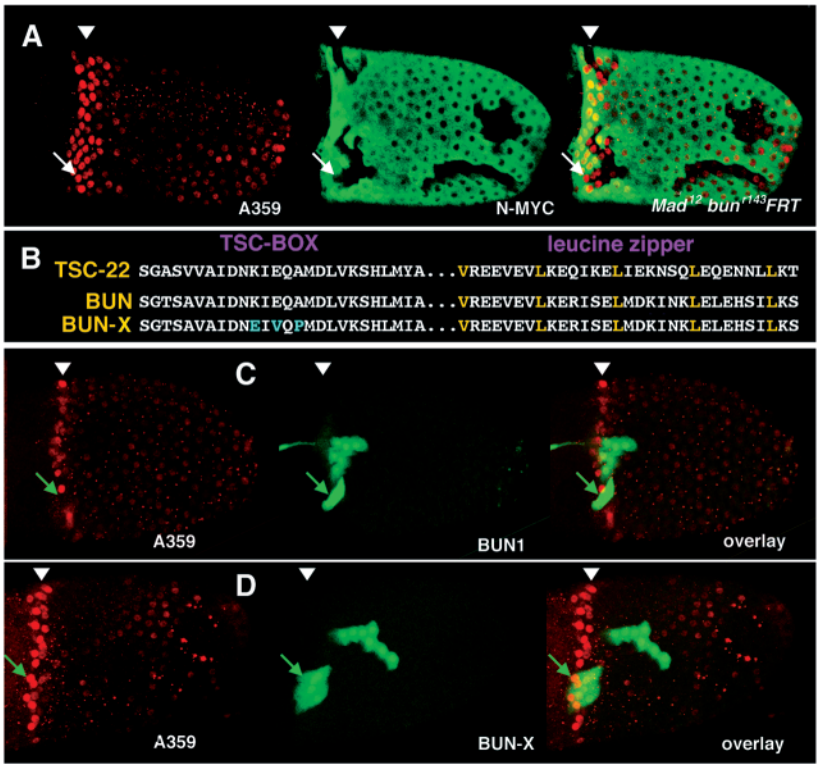

Fig. 5. DPP relieves *bunched* repression to induce anterior fates. Nuclear-β-galactosidase expressed from A359 is detected with rhodamine (red). (A) Reduced *bunched* bypasses the requirement for DPP to induce A359 expression. *Mad bunched* mutant clones located in the posterior had no effect on basal A359 expression. Anterior *Mad bunched* mutant clones in the presumptive centripetal migrating FC (white arrowhead) showed A359 expression levels similar to heterozygous neighbors (genotype *hs-FLP1; Mad*^{12} *bun*^{r143}*FRT 40A/ hs-NMYC FRT 40A; A359.1M3*). Mutant cells lack the MYC antigen, detected with fluorescein (green). (B) Sequences of UAS-BUNCHED derivatives. TSC-22 and BUN-1 share sequence homology including leucine zipper structure and TSC-box. BUN-X has three substitutions in the TSC-box (K_{60} to E, E_{62} to V, and A_{64} to P) but retains the leucine zipper domain. (C) Overexpression of BUN-1 is sufficient to block A359 expression. Flip-out overexpression of GAL4 and BUN-1 was detected using UAS-GFP (green). Anterior clones overexpressing BUN-1 led to cell autonomous repression of A359 (green arrow; genotype *hs-FLP1/actin5C>CD>GAL4; UAS-BUN-1/A359.1M3 UAS-GFP*). (D) Ectopic expression of BUN-X detected by UAS-GFP had no effect (green arrow; genotype *UAS-BUN-X/actin5C>CD2>GAL4; hs-FLP2; UAS-GFP A359.1M3*) on A359 expression at stage 10.

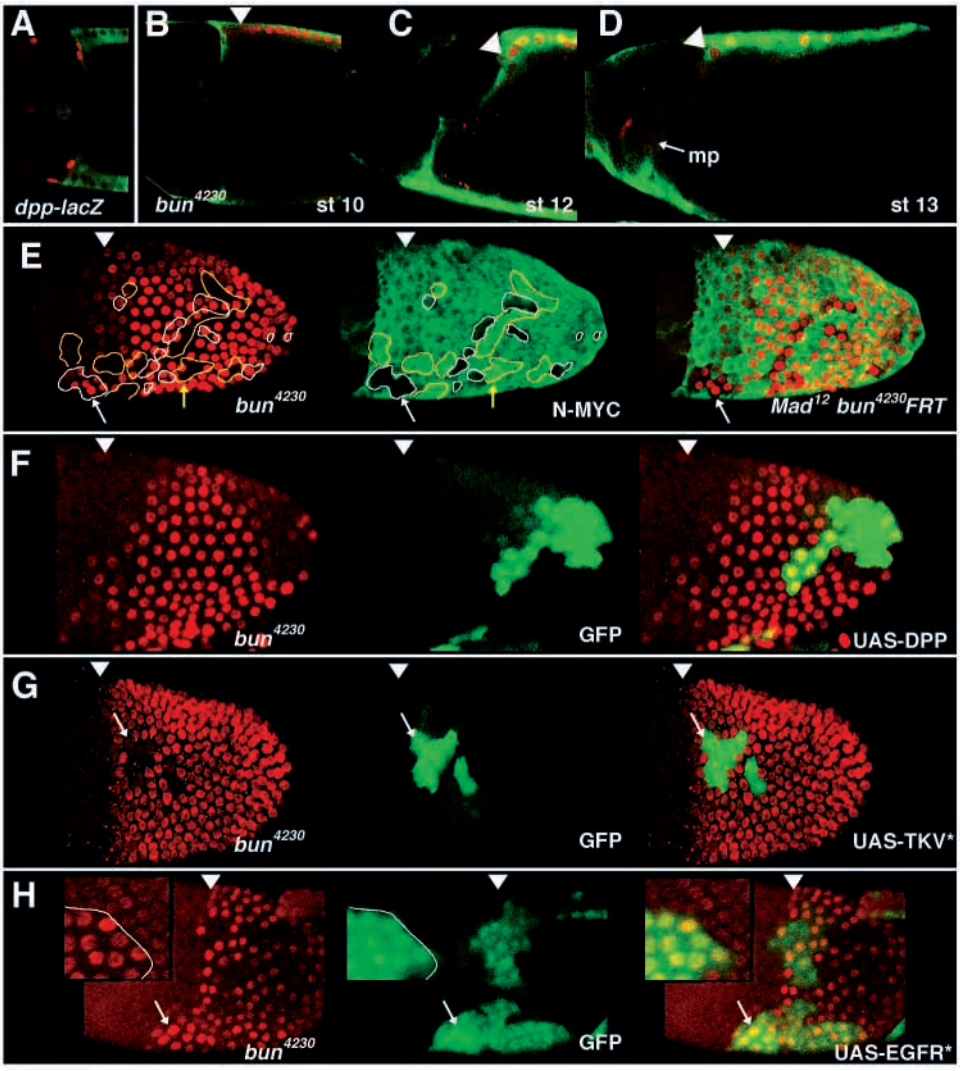

Fig. 6. The pattern of *bunched-lacZ* expression is achieved by DPP repression and EGF activation. (B-D) Confocal optical cross section shows *bunched-lacZ* (*bun*^{4230}) expression in cells adjacent to A359-expressing cells (marked by arrowhead); *dpp-lacZ* is shown for comparison (A). Nuclear-β-galactosidase expressed from *bunched-lacZ* or *dpp-lacZ* is detected with rhodamine (red). At later stages, *bunched-lacZ* expression is retained in dorsal-anterior FC; expression is strictly excluded from the operculum FC (arrowhead, B-H). (E) The DPP signal transducer *Mad* is required for *bunched-lacZ* repression. Egg chamber from female genetically *hs-FLP1; Mad*^{12} *bun*^{4230} *FRT40A/ N-MYC FRT40A*. *Mad* mutant clones (outlined in white) lack the MYC marker and carry two copies of *bun*^{4230}. Twin spot clones have two copies of the N-MYC marker (outlined in yellow). These cells do not carry *bunched-lacZ*; thus, they lack β-galactosidase (yellow arrow). *Mad bunched-lacZ* mutant cells show normal *bunched-lacZ* expression in the posterior FC. *Mad bunched-lacZ* double mutant cells (outlined in white) in the anterior FC ectopically expressed *bunched-lacZ* (white arrow). (F) DPP can repress *bunched-lacZ* expression in posterior FC. Egg chamber from female genetically *hs-FLP1/actin5C>CD>GAL4; bun*^{4230}*; UAS-dpp/UAS-GFP*. (F-H) Clones expressing GAL4 were detected by GFP fluorescence (green). Ectopic expression of DPP can repress *bunched-lacZ*, both in cells of the clone and in adjacent cells. However, not all cells that express DPP show repression of *bunched-lacZ*. (G) Expression of constitutively active TKV receptor (TKV-A) repressed *bunched-lacZ* only in cells marked by GFP (arrow). (H) Activated EGF-R is sufficient to misexpress *bunched-lacZ* in anterior FC. Flip-out clones expressing constitutively active EGF-R (UAS-EGFR*=λtop) showed increased expression of *bunched-lacZ*. Elevated *bunched-lacZ* levels were autonomous to the clone (inset), detected by GFP fluorescence (green), and occurred in both posterior and anterior (arrow) FC. Egg chamber from female genetically *UAS-λtop/actin5C>CD>GAL4; bun*^{4230}*; Sb hs-FLP/UAS-GFP*.

bun-lacZ expression in GFP-positive cells, even if these cells were adjacent to cells with ectopic repression of *bun-lacZ* (Fig. 6F). It seems likely that the availability of extracellular ligand is not simply determined by induction of gene expression.

To establish that repression of *bunched-lacZ* is a cell-autonomous response to DPP, we used the flip-out GAL4 system to create clones of cells that express constitutively active TKV receptor (TKV-A; Haerry et al., 1998). UAS-GFP-

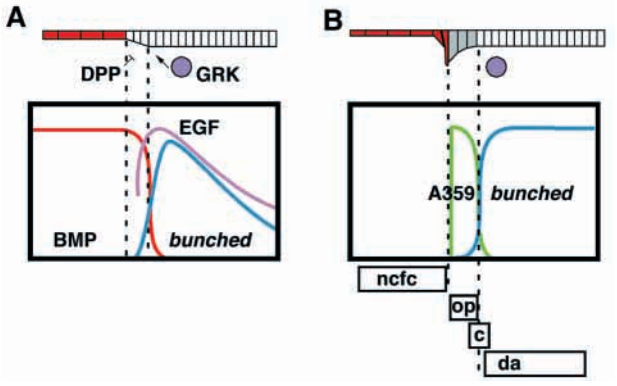

Fig. 7. BUNCHED is regulated negatively by DPP and positively by EGF to restrict the operculum fate. (A, top) Cross section of the dorsal follicular epithelium at stage 10A is depicted. DPP (red) is expressed in the nurse cell FC; the EGF ligand GRK is produced by the oocyte nucleus (purple). GRK patterns dorsal FC at this time. (A, bottom) Presumed levels (y-axis) of DPP/BMP (red), GRK/EGF (purple), and BUNCHED (blue) are presented along the FC epithelium (x-axis). (B, top) The dorsal follicular epithelium at stage 10B: DPP is expressed in the nurse cell FC and at the leading edge of centripetal migration (red); the centripetally migrating FC are gray. (B, bottom) Graphical representation of BUNCHED (blue) and A359 (green) expression, as in A. BUNCHED restricts the anterior fates marked by A359 expression, and sets the operculum boundary, marked by the collar. Different domains of cells give rise to specific eggshell structures, operculum (op), collar (c), dorsal appendages (da).

marked clones expressing TKV-A show cell autonomous repression of *bunched-lacZ*, regardless of size or position (*n*=15; Fig. 6G). Together, these data demonstrate that DPP signaling is necessary and sufficient to prevent *bunched-lacZ* expression in the anterior FC.

We previously reported that the peak of dorsal anterior *bunched-lacZ* expression is dependent on EGF signaling (Dobens et al., 1997). Another signal appears to maintain *bunched-lacZ* expression in the ventral posterior FC of late stage 10 egg chambers (LL Dobens and LA Raftery, unpublished data). We wished to determine whether EGF signaling could antagonize DPP signaling to induce ectopic *bunched-lacZ* expression. Clones of cells expressing constitutively active EGF receptor, *λtop* (Queenan et al., 1997), were induced using the flip-out GAL4 system. In the anterior FC, *λtop* expression was sufficient to induce cell-autonomous expression of bun^{4230} (*n*=5; Fig. 6H). Thus, elevated EGF-R signal can override the negative regulation by DPP signaling. We conclude that the sharp dorsal-anterior boundary for *bunched-lacZ* expression is set by the balance of positive signals through EGF-R and negative signals through TKV.

DISCUSSION

Members of the TGFβ superfamily play prominent roles in patterning both vertebrates and invertebrates. In particular, the bone morphogenetic proteins (BMPs), which include DPP, are required for the development of many tissues (reviewed in Hogan, 1996; Raftery and Sutherland, 1999). In some tissues, BMPs can function as morphogens, for they can induce multiple cell fates across a developmental field (reviewed in Neumann and Cohen, 1997; Whitman, 1998). In other tissues, BMPs appear to specify a distinct fate with a sharp boundary (e.g. in feather tract patterning in chickens, (Noramly and Morgan, 1998) and wing vein specification in flies (de Celis, 1997)). In vertebrates, BMPs often appear to oppose extracellular signals that activate receptor tyrosine kinases (RTKs) (Kretzschmer et al., 1997; Neubuser et al., 1997; Niswander and Martin, 1993). However, in *Drosophila*, both cooperative (e.g., Szüts et al., 1998) and antagonistic (e.g., Wappner et al., 1997) interactions have been observed. The follicle cells that surround the developing *Drosophila* oocyte provide a tractable system to examine interactions between BMP and RTK pathways. Here we provide evidence that *bunched* sets the boundary for the eggshell operculum, and demonstrate that the *bunched* expression pattern is set through negative regulation by DPP and positive regulation by EGF-R.

DPP responses define an anterior domain in the columnar follicle cells

To understand how DPP patterns follicle cells, we sought genes that respond to DPP signaling. Enhancer trap reporters at two loci were identified. The enhancer trap A359 is expressed in a narrow stripe of follicle cells adjacent to, but not overlapping with, DPP-expressing cells. Enhancer trap insertions in *bunched* are specifically repressed in anterior columnar FC and are expressed in the remaining columnar FC. The cells that do not express *bunched* will subsequently express A359 and then undergo centripetal migration.

Both A359 expression and *bunched-lacZ* repression are directed by DPP. Clones of anterior columnar FC that lack the DPP signal transducer MAD fail to express A359 and ectopically express *bunched-lacZ*. Ectopic DPP can drive ectopic A359 and repress *bunched-lacZ* in the columnar FC. Ectopic DPP ligand can cause *bunched-lacZ* repression in nearby cells, whereas the activated DPP type I receptor, TKV-A, represses *bunched-lacZ* cell autonomously. Thus, DPP expression in the nurse cell FC directs a domain of gene expression in the adjacent columnar FC. This domain becomes the centripetal migrating FC, and subsequently gives rise to the operculum on the dorsal side. DPP expression continues in the leading edge of the centripetal migrating FC (LEFC), perhaps acting to maintain the identity of these cells.

DPP represses BUNCHED to induce an anterior fate

Several lines of evidence indicate that *bunched* antagonizes operculum patterning. The operculum was expanded in eggs from females homozygous for a weak *bunched* allele. Consistent with this, egg chambers from these females had precocious and expanded expression of A359 in the follicle cells. BUN-1 expression was strongly reduced in the operculum-forming FC, and ectopic expression of BUN-1 was sufficient to block A359 expression in the anterior columnar FC. In addition, eggshells from *bunched* mutant females lacked the collar at the operculum boundary. These results suggest that the level of BUNCHED activity is critical to set the operculum boundary and to elaborate cell fates at this boundary.

Our previous work established that *bunched* is important for proper dorsal appendage formation (Dobens et al., 1997). Large dorsal anterior clones of *bunched* mutant FC result in dorsal appendages that are short and broadened with split ends. However, we found no evidence that *bunched* has an inductive activity in dorsal appendage formation. Rather, our data are consistent with a role for *bunched* as a repressor of the operculum fate. Perhaps ectopic activation of genes involved in operculum formation leads to aberrant dorsal appendage formation in *bunched* mutant clones.

Given that DPP promotes the operculum fate and *bunched* opposes it, we could genetically order their contributions. Anterior FC that are disabled for DPP signaling and defective in *bunched* function show normal levels of A359 expression. Thus, DPP indirectly promotes operculum formation by negative regulation of a negative regulator, BUNCHED.

Reduction of BUNCHED activity did not result in A359 expression throughout the columnar follicle cells (Figs 3D,F, 5A). This may be due to the use of a weak *bunched* allele for this analysis; low levels of BUNCHED may be sufficient to repress A359 in posterior FC. Alternatively, an additional signal may collaborate with DPP to direct A359 expression in the centripetal migrating FC. Isolation of null *bunched* alleles will resolve these possibilities.

The *bunched-lacZ* expression pattern is set by a balance of DPP and EGF signals

A role for *bunched* as an antagonist of operculum patterning correlates well with the strict exclusion of *bunched-lacZ* from the centripetal migrating follicle cells (CMFC), the anteriormost population of columnar FC. The dorsal CMFC undergo changes in shape that lead to the unique cell imprints in the operculum. Expression of *bunched-lacZ* occurs throughout the posterior FC, strongest over the dorsal anterior

FC. This expression pattern reflects the pattern of BUN-1 mRNA accumulation, which encodes the isoform that can block A359 expression. We conclude from these data that the dorsal anterior boundary of BUN-1 mRNA expression defines the future boundary of the operculum.

Previously, we demonstrated that GRK signaling through the EGF-R is necessary for normal *bunched-lacZ* expression in the dorsal anterior FC (Dobens et al., 1997). Here we show that ectopic expression of activated EGF receptor is sufficient to induce ectopic *bunched-lacZ* in the centripetal migrating FC. Conversely, DPP signaling is both necessary and sufficient to repress *bunched-lacZ* in columnar FC. Thus the dorsal anterior boundary of *bunched-lacZ* expression is set by a balance of positive EGF and negative DPP signals. DPP also sets the anterior boundary for *Broad-Complex* expression; however, the regulation of this gene by EGF signaling is more complex (Deng and Bownes, 1997).

In summary, we propose a model (Fig. 7) where the boundary for the operculum is set by the boundary of BUNCHED activity, which is positioned by opposing activity of DPP and EGF signals in the dorsal FC. Dorsal anterior FC are exposed to high levels of EGF ligands, GRK (Neuman-Silberberg and Schüpbach, 1993), SPITZ (Sapir et al., 1998; Wasserman and Freeman, 1998) and VEIN (Wasserman and Freeman, 1998), and thus have elevated *bunched* expression. High anterior DPP signaling represses *bunched* expression. The close apposition of these signals in the dorsal anterior FC creates a sharp boundary of *bunched* expression. BUN-1 functions to repress A359 and define the boundary to centripetal migrating FC fates, including the operculum. Our data indicate that the ventral operculum boundary is also set by *bunched*; however, another signal appears to promote ventral *bunched* expression at late stages (LL Dobens and LA Raftery, unpublished data). The normal operculum border is defined by the eggshell collar. This structure is lost as *bunched* activity is lowered, suggesting that the boundary of *bunched* expression may serve to further organize cell fates at the operculum boundary.

Although our data suggest that EGF signals antagonize operculum patterning, EGF signaling is essential for operculum formation. First, *grk* and EGF-R mutant eggs have no opercula (Schüpbach, 1987). Second, overexpression of activated EGF-R can result in operculum expansion, although interpretation of the specific phenotype is not straightforward (Queenan et al., 1997). Thus, we expect that DPP does not prevent all EGF-R-induced events in the operculum-forming FC. It is likely that EGF signaling is active in cells that lack BUNCHED activity, and that DPP inactivation of BUNCHED modifies the response of these cells to EGF signals. In cultured mammalian cells, RTK signaling can directly antagonize BMP signaling by preventing nuclear accumulation of Smad protein, offering a possible molecular mechanism for these interactions (Kretzschmer et al., 1997).

BUNCHED molecular function

bunched belongs to the TSC-22 family, a group of proteins that share both a leucine zipper and the adjacent, highly conserved TSC domain (Dobens et al., 1997; Treisman et al., 1995). TSC-22, the founding member of this family (Shibanuma et al., 1992), has a solution structure quite similar to that of bZIP transcription factors (Seidel et al., 1997). TSC-22 can bind to specific sequences in the promoter element from the C-type natriuretic peptide gene; these sequences drive TSC-22-dependent expression of a heterologous reporter (Ohta et al., 1996). Mutational analysis has shown that the TSC-domain is required for DNA binding and reporter gene regulation (Ohta et al., 1996). A recent report shows that TSC-22 can also act as a transcriptional repressor (Kester et al., 1999). Our data indicate that the A359 locus may be a direct target for negative regulation by BUNCHED. The sensitive A359 repression assay also shows that the TSC domain is critical to BUNCHED function: altering these amino acids makes BUN-1 inactive.

The role of *bunched* in other tissues is poorly understood. Embryos homozygous for *bunched* mutations die with morphological defects in the peripheral neurons (Kania et al., 1995) and subtle defects in cuticle pattern (L. L. D. and L. A. R., unpublished observations). *bunched* maternal effect phenotypes are pleiotropic, ranging from very early defects (J. S. P. and L. A. R., unpublished observations) to segmental defects in the embryonic cuticle (Perrimon et al., 1996). During eye development, *bunched* promotes photoreceptor differentiation, and shows genetic interactions with *dpp*, *wingless*, *hedgehog* and components of the EGF-R genetic pathway (Treisman et al., 1995). It remains to be determined whether *bunched* has a similar role throughout development, for example as an RTK target gene or a repressor of DPP target genes.

Here we show that *bunched* antagonizes DPP function in the follicle cells. This was surprising, for mutations in the *dpp* and *bunched* genes synergize to severely arrest eye development (Treisman et al., 1995). A mechanistic interpretation of this genetic interaction awaits better understanding of DPP functions during eye development. We note that the *bunched* eye phenotype is rescued by the BUN-2 transcript (Treisman et al., 1995), whereas BUN-1 is the antagonist of DPP in the FC. The BUN-2 transcript is expressed in the operculum-forming FC, raising the possibilities that this isoform has a distinct role, or that it is subject to post-transcriptional regulation. Further studies of the functions of the two isoforms will be needed to resolve these differences.

We propose here that the boundary to a DPP-induced fate in the follicle cells is set by transcriptional regulation of a downstream transcriptional repressor, BUNCHED. Recently, a similar role has been proposed for the gene *brinker* in setting threshold gene expression responses to DPP in the *Drosophila* wing (Campbell and Tomlinson, 1999; Jazwinska et al., 1999; Minami et al., 1999). Thus, DPP induction of gene expression through negative regulation of a negative regulator may be a common theme in development. Regulation of the expression of these key downstream repressors provides a powerful mechanism to modulate responses to DPP signaling.

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