**Drosophila** eggshell is patterned by sequential action of feedforward and feedback loops

Nir Yakoby1,*, Jessica Lembong1,* , Trudi Schüpbach2 and Stanislav Y. Shvartsman1,†

During Drosophila oogenesis, patterning activities of the EGFR and Dpp pathways specify several subpopulations of the follicle cells that give rise to dorsal eggshell structures. The roof of dorsal eggshell appendages is formed by the follicle cells that express Broad (Br), a zinc-finger transcription factor regulated by both pathways. EGFR induces Br in the dorsal follicle cells. This inductive signal is overridden in the dorsal midline cells, which are exposed to high levels of EGFR activation, and in the anterior cells, by Dpp signaling. We show that the resulting changes in the Br pattern affect the expression of Dpp receptor *thickveins* (*tkv*), which is essential for Dpp signaling. By controlling *tkv*, Br controls Dpp signaling in late stages of oogenesis and, as a result, regulates its own repression in a negative-feedback loop. We synthesize these observations into a model, whereby the dynamics of Br expression are driven by the sequential action of feedforward and feedback loops. The feedforward loop controls the spatial pattern of Br expression, while the feedback loop modulates this pattern in time. This mechanism demonstrates how complex patterns of gene expression can emerge from simple inputs, through the interaction of regulatory network motifs.

**KEY WORDS:** Cell signaling, Epithelial patterning, Feedforward control, Pattern formation, Drosophila

**INTRODUCTION**

Pattern formation in development relies on combinatorial interactions of a small number of signaling pathways, which can act either in parallel, converging on the enhancers of common target genes, or regulate each other at the level of signal generation, reception, transmission and interpretation (Martinez-Arias and Stewart, 2002). Biochemical and cellular mechanisms of signaling crosstalk become progressively characterized, but the dynamics of pathway integration at the tissue level remains poorly understood. Here we show how the evolutionarily conserved EGFR and BMP pathways interact in a network that controls epithelial patterning in Drosophila oogenesis, an established genetic model for studying the connection between signaling and developmental pattern formation (Deng and Bownes, 1998; Dobens and Raftery, 2000; Berg, 2005; Horne-Badovinac and Bilder, 2005).

The three-dimensional shell of the *Drosophila* egg is derived from the follicular epithelium in the developing egg chamber (Fig. 1A), (Spradling, 1993). Dorsal eggshell structures, the respiratory dorsal appendages and the operculum, are formed by the dorsoanterior follicle cells, which are patterned by Gurken (Grk), an EGFR ligand secreted from the oocyte (Schüpbach, 1987), and Dpp, a Bmp2/4-like molecule produced by the stretch follicle cells (Twombly et al., 1996; Dobens et al., 2000). Each dorsal appendage is formed by two groups of dorsolateral follicle cells (Dorman et al., 2004; Berg, 2005). The roof of the appendage is formed by cells expressing Broad (Br), a zinc-finger transcription factor (Deng and Bownes, 1997; Tzolovsky et al., 1999). The floor of the appendage is derived from the adjacent domain of cells expressing *rhomboid* (*rho*), which encodes an intracellular protease in the Drosophila EGFR pathway (Ruohola-Baker et al., 1993; Urban et al., 2001). Genetic approaches have established that the wild-type expression patterns of both *br* and *rho* depend on both EGFR and Dpp signaling (Neuman-Silberberg and Schüpbach, 1994; Deng and Bownes, 1997; Peri et al., 1999; Atkey et al., 2006). However, the origin of the dynamics of *br* and *rho* expression and their connection to eggshell patterning remained unclear.

Eggshell morphology can be dramatically affected by variations in the levels of Gurken and Dpp (Neuman-Silberberg and Schüpbach, 1994; Twombly et al., 1996; Dobens et al., 2000). Given this degree of sensitivity, it is unclear how these two inductive signals, which originate from two different tissues, coordinate their actions in order to establish the elaborate gene expression patterns and eggshell morphology. One mechanism for coordinating the actions of the EGFR and Dpp pathways may involve signaling crosstalk. Indeed, recent studies have shown that EGFR signaling influences the effects of Dpp by regulating the expression of inhibitors of Dpp signaling and have demonstrated that this type of pathway interaction is important for the formation of the Br expression domain (Chen and Schüpbach, 2006; Shravage et al., 2007).

Here we demonstrate that the patterning mechanism is considerably more complex. We found that, in addition to being a target of Dpp signaling, Br regulates the Dpp pathway in late stages of oogenesis by controlling the expression of the type I Dpp receptor *thickveins* (*tkv*). The Br-dependent changes in *tkv* expression lead to changes in the spatial pattern of Dpp signaling. As a result, the late phase of Dpp signaling in oogenesis has a clear dorsoventral polarity, in contrast to the early phase of Dpp signaling, which is uniform along the dorsoventral (DV) axis. We show that the early and late phases of Dpp signaling control different spatial domains of the *br* expression pattern. The early phase of Dpp signaling represses *br* in the anterior follicle cells, whereas the late phase of Dpp signaling limits the duration of *br* expression in the cells that form the roof of future dorsal appendages. We integrate these findings with the results of previous studies and propose a new model of eggshell patterning, in which the pattern of Br is established by the sequential action of feedforward and feedback loops.
MATERIALS AND METHODS

Genetics
The following stocks were used in this study: wild-type OreR, y w; cice fly (Jimenez et al., 2000), X7;28.20 (which contains four copies of P[w+ grk+] (Neuman-Silberberg and Schüpbach, 1993), EgfrGVT (Schüpbach, 1987), US-DS-Ad (a gift from J. Duffy), C2-Ga4 (Queenan et al., 1997; Goentoro et al., 2006b), D. pseudoobscura (a gift from V. Oogogozo and D. Stern). D. viridis, and D. phalerata (a gift from J. Duffy). The FLP/FRT mitotic recombination system (Xu and Rubin, 1993; Duffy et al., 1998) was used to generate clones of mutant follicle cells marked by the absence of GFP. The following genotypes were used to analyze the effects of the EGFR pathway: a null allele of Rsc (Ruohola-Baker et al., 1993; Sapir et al., 1998; Wasserman and Schüpbach, 1993; Pai et al., 2000; Goentoro et al., 2006a). The later stock was used to the effects of the BMP pathway used the following alleles: Med1 (Chen and Schüpbach, 2006), Mad12 (a gift from R. Padgett). Flies were grown on agar cornmeal medium; all crosses were done at 23°C. In Br overexpression experiments, the UAS-Br-Z1 construct (a gift from L. Riddiford) was driven by the Gal80-Gal4, CY2-Gal4 expression system (Suster et al., 2004). Mosaic analysis of the effects of br cells were done with the brFRT FRT40A; e22c-Ga4 UAS-FLP flies (Chen and Schüpbach, 2006).

In situ hybridization
The following primers were used to amplify part of the tkv gene from D. phalerata cDNA: forward primer 5'-ACATCATGAGCATGCATCCT-3' and a reverse primer 5'-CGGCATGCATATCATCAAAG-3' in a PCR (iCycler, BioRad). The product (933 bp) was cloned using a StrataClone PCR Cloning Kit (Stratagene). The clone was sequenced (GeneWiz) and BLASTed against the D. melanogaster Genome (FlyBase). In situ hybridization was carried out as previously described (Wang et al., 2006), but without the RNase digestion step. The digoxigenin-labeled RNA probes were made from a cdna clone of br-Z2 (provided by W-M. Deng), tkv (provided by M. O’Connor), rho (Ruhola-Baker et al., 1993), and the tkv clone from D. phalerata.

Immunofluorescence and microscopy
Dissection and fixation of ovaries was done as described elsewhere (Pacquelet and Rorth, 2005) Primary antibodies: mouse anti-Brd (25E9.D7; 1:1000, DSHB), rabbit anti-phosphorylated-Smad1/5/8 (1:3000, generous gift from D. Vasiiliauskas, S. Morton, T. Jessell and E. Laffter), rabbit or mouse anti-GFP (1:2000, Molecular Probes and Chemicon International, respectively), rabbit or mouse Anti-HA monoclonal antibodies (1:2000, Chemicon) and Hoechst dye to stain the nuclei (1:10,000). Secondary antibodies: Alexa Fluor and Oregon Green (1:2000, Molecular Probes). Images were taken with a PerkinElmer RS3 Spinning Disk Confocal microscope and the Nikon Eclipse E800 compound microscope. Images were processed with ImageJ (Rasband, 1997-2006) and Photoshop (Adobe Systems, San Jose, CA). ESEM images were taken as described elsewhere (Yakoby et al., 2005).

RESULTS
The spatial pattern of Dpp signaling acquires DV polarity in late stages of egg development
At stages 9-10 of oogenesis, EGFR is activated in a broad DV gradient (Fig. 1E,F). In older egg chambers the pattern of EGFR signaling is restricted dorsally and split along the dorsal midline (Fig. 1G). This transition is relatively well understood and depends on the change in the identity and the source of the EGFR-activating ligand. The early pattern is mediated by Gurken, secreted from the dorsoanterior cortex of the oocyte (Neuman-Silberberg and Schüpbach, 1993; Pai et al., 2000; Goentoro et al., 2006a). The later pattern is mediated by Spitz, an EGFR ligand secreted by the two L-shaped stripes of the follicle cells on either side of the dorsal midline (Ruhola-Baker et al., 1993; Sapir et al., 1998; Wasserman and Freeman, 1998; Peri et al., 1999).

In contrast to the dynamic pattern of EGFR activation, the pattern of Dpp signaling is viewed as a static anteroposterior (AP) gradient that persists through all stages of eggshell patterning (Peri and Roth, 2000; Berg, 2005; Shravage et al., 2007). Before stage 10 of oogenesis, such a pattern can be readily explained by a model in which the anteriorly secreted Dpp acts through receptors that are...
uniformly expressed throughout the follicular epithelium (Peri and Roth, 2000; Jekely and Rorth, 2003). Later, however, the spatial pattern of the type I Dpp receptor tkv acquires a clear DV polarity (Mantrona et al., 1999). In stage 10B egg chambers, tkv is expressed in a dorsoventral pattern with two lateral patches on both sides of the dorsal midline (Fig. 1B-D).

As tkv is essential for Dpp signaling in multiple stages of fly development, we asked whether this change in tkv expression could lead to the corresponding change in the pattern of the Dpp pathway activation. Consistent with this prediction, we found that the pattern of phosphorylated Mad (P-Mad), which is uniform along the DV axis before stage 10 (Fig. 1H), acquires a clear DV polarity in older egg chambers. At stage 10B P-Mad was detected in approximately five dorsal cell rows and in one to two ventral cell rows, respectively (Fig. 1I). At stages 11 and 12, the pattern of Dpp signaling split along the dorsal midline and shifted posteriorly, which correlates with the corresponding change in the tkv pattern (Fig. 1C). Thus, after stage 10B of oogenesis, the activation patterns of both the EGFR and Dpp pathways are split along the dorsal midline (Fig. 1M).

**The AP-to-DV transition in the pattern of Dpp signaling is conserved across species**

In contrast to recently published observations (Shravage et al., 2007), our results establish that the pattern of Dpp activation undergoes a clear transition from the purely AP to the DV pattern in late stages of oogenesis. Remarkably, we found that this transition is conserved in other fruit fly species (Fig. 2A). The early spatial patterns of P-Mad were uniform along the DV axis in egg chambers of *D. pseudoobscura, D. phalerata* and *D. virilis* (Fig. 2B,E,H). Later, however, the P-Mad pattern in each of these species acquired a clear DV polarity, just as it does in *D. melanogaster* (Fig. 2C,F,I-K). For instance, in *D. phalerata*, the anterior ring of P-Mad in mid-oogenesis underwent a transition to a ventral band of P-Mad in older egg chambers (Fig. 2I-K). Importantly, this transition closely followed a similar transition in the expression of tkv in this species (Fig. 2L-N).

Based on these observations, we made a number of predictions regarding the origin and significance of the Dpp signaling dynamics in mid- to late stages of oogenesis. First, based on the correlation between the spatial patterns of P-Mad and tkv, we predicted that the DV pattern of tkv controls the spatial pattern of Dpp signaling. Second, as the DV polarity in oogenesis is due to EGFR signaling, we predicted that the pattern of tkv depends on EGFR signaling. Finally, based on the evolutionarily conserved nature of AP-to-DV transition in the pattern of Dpp signaling, we predicted that this transition is functionally significant for eggshell patterning. We have used a combination of gain- and loss-of-function approaches to directly test each of the predictions in *D. melanogaster*.

**The spatial pattern of tkv regulates the spatial pattern of Dpp signaling and is sensitive to EGFR signaling levels**

To test whether tkv is essential for Dpp signaling in the follicular epithelium, we generated GFP-marked clones of tkv cells and monitored the resulting change in the P-Mad pattern. We found that P-Mad signal disappeared in clones of tkv cells, confirming the essential role of tkv for Dpp signaling (Fig. 3A-C). Thus, the midline repression of tkv at stage 10 of oogenesis (Fig. 1C), can account for the corresponding repression in the wild-type pattern of P-Mad (Fig. 1J).

Based on previous studies of eggshell patterning, the midline gap in the tkv pattern corresponds to the highest levels of EGFR activation in the follicular epithelium, whereas the dorsolateral patches correspond to the intermediate levels of EGFR activation (Neuman-Silberberg and Schüpbach, 1994; Goentoro et al., 2006a). Based on this correlation between the estimated gradient of EGFR activation and the wild-type tkv pattern, we hypothesized that high levels of EGFR signaling downregulate tkv, while intermediate levels of EGFR signaling induce tkv expression. The midline repression could be due to Pointed, which is activated by high levels of EGFR signaling and represses EGFR targets (Fig. 3D) (Morimoto et al., 1996; Deng and Bownes, 1997; Wasserman and Freeman, 1998; Ward et al., 2006).

This model predicts that a stronger and broader pattern of EGFR activation should increase the distance between the dorsolateral patches of tkv, and that a reduced gradient of EGFR activation can eliminate the dorsolateral patches in the tkv pattern (Fig. 3D). Both these predictions are supported by in situ hybridization analysis of tkv expression in mutants with quantitative variations in EGFR signaling. The distance between the two dorsolateral patches of tkv were abolished in the egg chambers with a hypomorphic allele of tkv (Fig. 3F). The P-Mad pattern closely followed these transitions (Fig. 3G,H), once again demonstrating that Dpp signaling is controlled by the spatial pattern of tkv.

**Correlation between the dynamics of Dpp signaling and Br expression**

In the next set of experiments, we used Br, a transcription factor expressed in the cells that form the roof of future dorsal appendages, to study the patterning effects of Dpp signaling. Br is
initially expressed at low levels throughout the oocyte-associated follicular epithelium; it is then repressed in the anterior and dorsal midline cells (Fig. 4A) (Deng and Bownes, 1997). The P-Mad domain borders, but does not overlap the Br domain at this stage (Fig. 4A). At later stages of oogenesis, Br is expressed at high levels in two dorsolateral patches of the follicle cells, called the ‘roof’ cells (Fig. 4B,C). High levels of Br in the roof cells became clear at stage 10B of oogenesis. At this stage, the P-Mad domain expanded posteriorly and overlapped the most anterior row of Br-expressing cells (Fig. 11 and Fig. 4B). From stage 11 and onward, the patterns of P-Mad and Br overlapped quite significantly (Fig. 4C).

Until stage 10B of oogenesis, the dynamics of br transcript and Br protein levels appear to be highly correlated in space and time (Fig. 4D-F). Low levels of br transcript were detected in all follicle cells before stage 9; br was then repressed in the dorsal midline and anterior cells, and began to emerge in the roof cells. At later stages, however, br is downregulated in the roof cells (Fig. 4G) (Deng and...
Bownes, 1997). Thus, while the levels of Br protein in the roof cells are sustained, the expression of br transcript in these cells is transient, and can be viewed as a pulse in time. We will return to this difference in our analysis of the connection between Dpp signaling and br.

**Dpp signaling represses Br in the anterior follicle cells**

Previous studies of the Dpp effects on Br expression in oogenesis led to two different models. Results of experiments with the manipulation of the Dpp levels or intracellular inhibitors of Dpp signaling suggested that Dpp acts as a repressive signal (Deng and Bownes, 1997; Dequier et al., 2001; Chen and Schüpbach, 2006). However, studies of Br pattern in egg chambers with clones of tkv- and Medea+ (Med+, a co-Smad essential for Dpp signal transduction) cells concluded that Dpp signaling provides a positive input for Br expression (Peri and Roth, 2000; Shravage et al., 2007). Given these conflicting results and the dynamic character of Br expression, we revisited the effect of Dpp on Br.

The sign of the effect of Dpp signaling on Br can be deduced from changes of the Br pattern in response to the local perturbations of the Dpp pathway. For example, if Dpp acts as a repressive signal, then negative perturbations of Dpp signaling are expected to lead to ectopic Br expression in the anterior cells. To test this prediction, we generated the GFP-marked clones of cells lacking Medea, and examined their effect on the expression pattern of Br. We found that anterior Med+ clones generated cell-autonomous ectopic Br expression (Fig. 4H-J). We found similar results in experiments with anterior clones tkv- cells (see Fig. S1 in the supplementary material) and in the egg chambers expressing tkv-RNAi construct (see Fig. S2 in the supplementary material) (Crickmore and Mann, 2006; Dietzl et al., 2007). Based on this, we concluded that Dpp signaling represses Br in the anterior follicle cells. This is consistent with the anticorrelated patterns of Br and P-Mad in these cells (Fig. 4A,B).

**Dpp signaling limits the duration of br expression in the roof cells**

In contrast to their clear effect in the anterior of the egg chamber, Med- and Mad- clones did not affect Br expression in the roof cells (Fig. 4K-P). Furthermore, we observed no defects in Br expression in the roof cells in egg chambers where the Dpp pathway was uniformly inhibited by overexpressing Dad, an inhibitory Smad (Fig. 5B). The only change in the Br pattern in this background amounted to its expansion to the anterior boundary of the follicle epithelium (Fig. 5B). These observations would be consistent with the model in which the effect of Dpp is repressive. Indeed, removal of repressor in the region that already expresses Br (roof cells) can only increase the level of expression, and could not be detected on the background of already strong Br expression level.

The appearance of P-Mad in the roof cells correlates with the disappearance of br transcript in this region (Fig. 4C,G). Based on the repressive effect of the Dpp pathway in the anterior cells, we hypothesized that the late (DV) phase of Dpp signaling is responsible for the downregulation of br transcript in the roof cells. This predicts that in the absence of Dpp signaling, the transient pulse-like pattern of br expression in the roof cells should be transformed into a sustained temporal pattern (Fig. 5A). In agreement with this prediction, we found that in egg chambers with uniformly inhibited Dpp signaling, br was still expressed at high levels in the roof cells at stage 11 of oogenesis, when it is already abolished in the wild type.

**Fig. 5. Dpp signaling limits the duration of br expression in the roof cells.** (A) Schematic of the dynamics of br levels in the roof cells in the wild type (dark gray). We predicted that the pulse-like dynamics of br expression in the roof cells should become sustained in the absence of Dpp signaling (light gray). (B) Uniform expression of Dad, the inhibitory Smad, changes the spatial pattern of Br protein: Br is ectopically expressed in the anterior cells. (C) At the same time, the duration of br expression in the roof cells is increased, compared with the wild-type pattern of br expression (Fig. 4G). (D) Changes in the spatial pattern of Br in the anterior cells generate a predictable change in the expression of rho, which was previously shown to be repressed by Br. (E) Schematic of rho (blue) and Br (red) patterns. (F) Patterning defects in egg chambers with uniformly inhibited Dpp signaling lead to defects in eggshell morphology: EM image of Dad overexpression eggshell shows flat dorsal appendages and reduced operculum size. (G) Wild-type rho expression; (H) schematic of wild-type rho (blue) and Br (red) patterns; (I) wild-type eggshell morphology. In B, C, D, G anterior is to the left; the dorsal midline is marked by an arrowhead; yellow dashed lines mark the anterior boundary of the oocyte. DA, dorsal appendages; op, operculum.

**Fig. 6. EGFR represses Br in the midline and is required for Br expression in the roof cells of Drosophila.** (A-D) Experiments with genetic mosaics with GFP-marked clones of ras- cells reveal region-specific effects of EGFR signaling on Br expression. The dorsal midline is marked by an arrowhead in all images. (A) Merged image of Br expression (red) and GFP (green). (B) Midline ras- clones induce cell-autonomous ectopic expression of Br. (C, D) Lateral clones of ras- cells lead to cell-autonomous loss of Br expression in the roof cells. (E) A model proposed for the regulation of Br expression by EGFR: the spatial pattern of Br is established by the feedforward loop controlled by EGFR signaling. Br is induced in a wide dorsal domain. This effect is overridden in the dorsal midline cells, where Pointed, induced by high levels of EGFR signaling, represses Br.

In the supplementary material) (Crickmore and Mann, 2006; Dietzl et al., 2007). Based on this, we concluded that Dpp signaling represses Br in the anterior follicle cells. This is consistent with the anticorrelated patterns of Br and P-Mad in these cells (Fig. 4A,B).
Furthermore, in this background the br pattern expanded at the expense of the cells that would normally express rho and contribute to the floor of dorsal appendages (Fig. 5D,E) (Ward et al., 2006). This change in the two-dimensional arrangement of cell fates led to eggshells with greatly reduced operculum and deformed dorsal appendages (Fig. 5F-I). This shows that uniform inhibition of Dpp signaling in mid-stages of oogenesis does not cause a developmental delay, and that the observed changes of br expression reflect the altered pattern of Dpp signaling. Uniform expression of tkv-RNAi (Crickmore and Mann, 2006; Dietzl et al., 2007) led to similar effects: we observed longer persistence of br in the roof cells and ectopic br in the anterior of the follicular epithelium, and defects in dorsal eggshell structures (see Fig. S2 in the supplementary material). A significant fraction of tkv-RNAi eggshells had ectopic dorsal appendages (see Fig. S2 in the supplementary material), consistent with the model in which br marks the roof of dorsal appendages (Berg, 2005). Taken together, these data show that Dpp represses Br in the anterior and limits the duration of br expression in the roof cells, and that this mode of regulation is important for proper eggshell patterning.

**Midline repression of Br is controlled by EGFR and independent of Dpp signaling**

We found that negative perturbations of the Dpp pathway did not affect Br repression in the midline cells. Specifically, clones of tkv, Med, and uniform expression of Dad did not generate ectopic Br in the midline (Fig. 4K-P, Fig. 5B,C). This shows that the midline repression of Br is independent of Dpp signaling. The midline repression of Br was attributed to Pointed, which is activated by high levels of EGFR signaling (Deng and Bownes, 1997). At the same time, the induction of Br in the roof cells is also dependent on EGFR signaling (Deng and Bownes, 1997; Atkey et al., 2006). This suggests that high levels of EGFR signaling repress, whereas intermediate levels of EGFR activate, Br expression. This model agrees with the results of our own clonal analysis experiments. Using genetic mosaics with clones of cells lacking ras, which is essential for EGFR signaling, we found that EGFR affects Br in a region-specific manner: the midline clones of ras cells led to ectopic expression of Br, while ras clones in the dorsolateral cells greatly reduced Br expression (Fig. 6A-D). Taken together with our analysis of the effects of Dpp signaling on Br (Fig. 4H-P), these results suggest that the rising phase of br expression in the roof cells is due to EGFR signaling, whereas its shutdown is due to Dpp signaling.

**Br expression in the roof cells is controlled by a negative-feedback loop**

The results of the previous sections suggest that EGFR signaling can induce both br and its repressor (Dpp signaling) in the roof cells (Fig. 3D, Fig. 6E). Two models can be formulated based on these results. In one model, the expression of br would be controlled by an incoherent feedforward loop, a network in which the input induces both the target and its repressor in the roof cells (Alon,
In this case, the input is EGFR signaling, the target is br, and its repressor is Dpp signaling in the roof cells. Alternatively, br expression in the roof cells could be controlled by a negative-feedback loop, whereby Br, activated by EGFR signaling, would induce tkv and Dpp signaling, which would then downregulate br. Note that although both of these networks are consistent with the pulse-like dynamics of br in the roof cells (Fig. 3 and Fig. 5A), only the negative-feedback network provides a natural way for generating an ordered induction of br and its repressor (Dpp signaling).

To discriminate between these two models, we tested whether tkv expression and Dpp signaling in the roof cells are controlled by Br. For this we overexpressed Br in the follicular epithelium and examined the effect on the spatial pattern of Dpp signaling and tkv expression (Fig. 7M). As a result, we found ectopic Dpp signaling in the anterior follicle cells, including those in the dorsal midline (Fig. 7A-C), probably due to the ectopic expression of tkv (Fig. 7D and see Fig. S3 in the supplementary material). This suggests that Br expression in the dorsal follicle cells is sufficient for the induction of tkv. In a complementary experiment, we observed that P-Mad signaling disappeared in GFP-marked clones of br– cells (Fig. 7E-J). Furthermore, we found systematic disruptions in the spatial pattern of tkv in egg chambers with clones of br– cells (Fig. 7K). This qualitative change of a very robust two-patched wild-type pattern of tkv expression strongly suggests that Dpp signaling depends on the Br-mediated expression of tkv (Fig. 7L). In combination with the repressive effect of Dpp signaling on br expression, these results provide strong evidence for the negative-feedback control of br levels in the roof cells (Fig. 8A). At the same time, these results show that the effects of the EGFR activation on the pattern of tkv expression and Dpp signaling (Fig. 3) are mediated by Br.

**DISCUSSION**

Our results provide new insights into the dynamics and function of the Dpp pathway in oogenesis. First, we demonstrate that, contrary to the current model of *Drosophila* eggshell patterning (Berg, 2005; Shravage et al., 2007), the pattern of Dpp signaling in oogenesis is not static, and undergoes a clear transition from purely AP to DV pattern in late stages of eggshell patterning. This transition reflects the change in the expression of the type I Dpp receptor and is conserved in fly species separated by more than 40 million years of evolution. Second, we show that the early and late patterns of Dpp signaling control the dynamic pattern of br, a transcription factor expressed in the roof of future dorsal appendages. While the AP phase of Dpp signaling represses br in the anterior region of the egg chamber, the DV phase of Dpp signaling limits the duration of br expression in the roof cells. Third, we establish that, in addition to being regulated by Dpp, Br actively controls Dpp signaling, thus regulating its own repression via a negative-feedback loop.

**The Br expression pattern is established by sequential action of feedforward and feedback loops**

Our results, together with the previously published data, lead to a new model for the dynamics of Br expression in the roof cells (Fig. 8A). Within the framework of this model, the rising phase of Br expression is due to an incoherent feedforward loop, a network in which the input activates both the target and its repressor (Alon, 2007). In this case, the feedforward loop, formed by EGFR, Pointed, and Br, determines the spatial pattern of Br (Fig. 6E). This pattern is then modulated in time by a negative-feedback that depends on the Br-mediated increase of tkv expression and Dpp signaling. The feedforward part of the model (outlined in blue) is supported by the previously published gain- and loss-of-function experiments with Pointed and EGFR signaling (Morimoto et al., 1996; Deng and Bownes, 1997; Yamada et al., 2003), and by our analysis of Br expression in ras– mosaics (Fig. 6). The negative-feedback loop (outlined in red) is supported by the correlation of patterns of Br, Tkv, and P-Mad (Fig. 3), by the previously published experiments with manipulation of the levels of Dpp (Deng and Bownes, 1997; Dequier et al., 2001), by our analysis of Br protein and br transcript in the Dpp pathway loss-of-function experiments (Fig. 4H-P, Fig. 5), and by the effects of br– clones and Br overexpression on tkv and Dpp signaling (Fig. 7).

**Dynamics of two-dimensional patterning in the model**

We distinguish four phases in the dynamics of the Br pattern (Fig. 8B). (1) Low levels of Br before stage 9 of oogenesis are independent of EGFR signaling and insensitive to repression by Dpp. (2) Following the formation of the DV gradient of EGFR activation, Br is repressed in the midline and in the dorsosanterior cells. The midline repression is due to Pointed, a transcription factor induced by high levels of EGFR activation in the dorsal midline (Morimoto et al., 1996; Deng and Bownes, 1997; Yamada et al., 2003). The dorsosanterior repression is due to the early phase of Dpp signaling, which reflects the anterior secretion of Dpp and uniform expression of Tkv (Peri and Roth, 2000; Jekely and Rorth, 2003).

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**Fig. 8. Model for the dynamic regulation of Br by EGFR and Dpp pathways.** (A) A revised model of Br regulation by EGFR and Dpp signaling. The rising phase of Br expression in the roof cells is due to a feedforward loop activated by EGFR signaling (blue). The shutdown of Br expression in the roof cells is due to a negative-feedback loop (red), which is formed by Br and Dpp signaling. See text for details. (B) Schematic diagram of the spatial and temporal patterns of the main components in the model. See text for details.
(3) Levels of Br begin to rise in the roof cells. Changes in the Br pattern have two effects on the spatial pattern of Dpp signaling: higher levels of Br lead to higher levels of tkv in the roof cells. Second, the dorsoanterior and midline repression of Br generates a corresponding repression of tkv. (4) As a result, the anteriorly produced Dpp can diffuse over the ‘Tkv-free’ area to the roof cells. A combination of the arrival of the anteriorly produced ligand and a higher level of receptor expression leads to a higher level of Dpp signaling in the roof cells and subsequent repression of br. Another layer of regulation is provided by Brk, a transcriptional repressor of Dpp signaling, which is induced by Gurken and repressed by Dpp signaling in the dorsal follicle cells. Brk antagonizes the repressive effect of Dpp in the roof cells until the level of Dpp signaling in the roof cells becomes high enough to repress Brk expression (Chen and Schüpbach, 2006; Shraige et al., 2007).

The network characterized in our study can interact with a number of previously discovered feedback loops (Queenan et al., 1997; Wasserman and Freeman, 1998; Ghiglione et al., 1999; Reich et al., 1999; Peri and Roth, 2000; Ward et al., 2006). For instance, Argos, which provides negative-feedback control of EGFR signaling in the dorsal midline, is a potential target of Dpp signaling (Queenan et al., 1997; Wasserman and Freeman, 1998; Klein et al., 2004). Future work is required to explore the extent to which this feedback loop, which had been proposed to affect dorsal midline patterning, interacts with the mechanism established in this paper.

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