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Feedback control of the EGFR signaling gradient: superposition of domain-splitting events in *Drosophila* oogenesis

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The morphogenesis of structures with repeated functional units, such as body segments and appendages, depends on multi-domain patterns of cell signaling and gene expression. We demonstrate that during *Drosophila* oogenesis, the two-domain expression pattern of Broad, a transcription factor essential for the formation of the two respiratory eggshell appendages, is established by a single gradient of EGFR activation that induces both Broad and Pointed, which mediates repression of Broad. Two negative-feedback loops provided by the intracellular inhibitors of EGFR signaling, Kekkon-1 and Sprouty, control the number and position of Broad-expressing cells and in this way influence eggshell morphology. Later in oogenesis, the gradient of EGFR activation is split into two smaller domains in a process that depends on Argos, a secreted antagonist of EGFR signaling. In contrast to the previously proposed model of eggshell patterning, we show that the two-domain pattern of EGFR signaling is not essential for specifying the number of appendages. Thus, the processes that define the two-domain patterns of Broad and EGFR activation are distinct; their actions are separated in time and have different effects on eggshell morphology.

KEY WORDS: Feedback, Feedforward, EGFR, Argos, Sprouty, Kekkon-1, Rhomboid, Pattern formation, Oogenesis

INTRODUCTION

The *Drosophila* eggshell is an elaborate structure that protects the embryo and mediates its interaction with the environment (Hinton, 1969; Spradling, 1993). It is derived from somatic follicle cells, arranged in an epithelial layer that envelops the developing egg chamber (Berg, 2005; Horne-Badovinac and Bilder, 2005). A subset of follicle cells patterned by the highly conserved EGFR pathway forms two respiratory eggshell appendages, also called dorsal appendages (DAs). Their specification is initiated when the TGFα-like ligand Gurken (GRK) is secreted from the dorsal anterior cortex of the oocyte and signals through EGF receptors on the neighboring follicle cells (Chang et al., 2008; Neuman-Silberberg and Schupbach, 1993; Neuman-Silberberg and Schupbach, 1994; Queenan et al., 1997). The resulting gradient of EGFR activation controls a number of transcription factors, signaling molecules and effector genes required for eggshell morphogenesis (Cavaliere et al., 2008; Dobens and Raftery, 2000; Wu et al., 2008; Yakoby et al., 2008a). Several other pathways, including Decapentaplegic (DPP) and Notch, are also involved in this process (Deng and Bownes, 1997; Twombly et al., 1996; Ward et al., 2006), but their role is secondary to that of the EGFR pathway as the dorsal eggshell structures are completely abolished in the absence of GRK (Schupbach, 1987).

The fate map for the formation of the dorsal eggshell structures consists of three domains (Berg, 2005). Spanning the dorsal midline is a cusp-like region of cells that contributes to the future operculum (Ward and Berg, 2005). At the lateral boundaries of this region are two L-shaped stripes of cells that form the floor (lower part) of the future appendages; these cells are marked by the expression of *rhomboid* (*rho*), a gene that encodes an intracellular protease that processes Spitz, another EGFR ligand (Ward and Berg, 2005; Wasserman and Freeman, 1998). Adjacent to each of the two floor domains is a group of cells that express the zinc-finger transcription factor Broad (BR) and form the roof (upper part) of the appendages (Deng and Bownes, 1997; Ward and Berg, 2005).

The sizes and shapes of the midline, floor and roof cell domains are regulated by EGFR signaling: increasing the level of the oocyte-derived GRK moves the floor cell domains further apart and leads to eggshells with widely spaced appendages (Neuman-Silberberg and Schupbach, 1994). Eggshells from mutants with a hypomorphic allele of *Ras85D* (*Ras85D*$^{\Delta C40B}$), which is essential for EGFR signal transduction, have a single appendage and a single, dorsally placed domain of BR in the follicular epithelium (James et al., 2002; Schnorr and Berg, 1996). The mechanism of GRK-mediated eggshell patterning has been the subject of intense research over the past two decades, but is still not completely understood. One of the central questions is the relationship between the shape and amplitude of the EGFR signaling gradient and the spatial arrangement of the cell fates that contribute to the dorsal eggshell structures.

In 1998, Wasserman and Freeman suggested that the induction of the DAs relies on feedback control of the single-peaked gradient of EGFR activation by GRK (Wasserman and Freeman, 1998). The mechanism was based on the discovery that GRK induces two autocrine feedback loops in the follicle cells. The first feedback loop, based on the activation of *rho*, amplifies EGFR signaling (Lee et al., 2001; Ruohola-Baker et al., 1993; Wasserman and Freeman, 1998). The second feedback loop, based on the induction of *argos*, which encodes a secreted antagonist of EGFR signaling (Freeman et al., 1992; Klein et al., 2004), was proposed to split the EGFR signaling gradient into two smaller domains that define the two disjoint groups of appendage-producing follicle cells (Wasserman and Freeman, 1998).

Since the formulation of this mechanism, two other inhibitors of EGFR signaling, Kekkon-1 (KEK1) (Ghiglione et al., 2003; Ghiglione et al., 2002; Ghiglione et al., 1999) and Sprouty (STY) (Casci et al., 1999; Hacohen et al., 1998; Reich et al., 1999), have

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been identified as being involved in eggshell patterning. Both are induced by GRK in the region of the follicular epithelium that partially overlaps with the domain of *argos* expression. Thus, three different negative-feedback loops control EGFR signaling, but their relative contributions to eggshell patterning remain unclear. For example, removal of *argos* has been reported to lead to a loss of the dorsal midline cell fate and to a single DA (Wasserman and Freeman, 1998), whereas removal of *kek1* has an opposite effect, leading to eggshells with an increased midline domain and two DAs (Ghiglione et al., 1999).

Until now, the effects of EGFR feedback regulators on eggshell patterning have been evaluated only on the basis of their effects on the final eggshell morphology, i.e. on the number of appendages and the distance between them (Ghiglione et al., 1999; Reich et al., 1999; Wasserman and Freeman, 1998). Here we explore their effects more directly, using BR as a marker of the DA cell fate and phosphorylated MAPK as a reporter of EGFR activation (Astigarraga et al., 2007; Dammai and Hsu, 2003; Dorman et al., 2004; Gabay et al., 1997; James and Berg, 2003; Kagesawa et al., 2008; Peri et al., 1999; Tzolovsky et al., 1999). Based on the extents to which *argos*, *rho*, *kek1* and *sty* influence the dynamics of BR expression and EGFR signaling, we conclude that feedback loops do not directly determine the number of appendages, but instead control the size and position of the appendage primordia.

The number of appendages, which equals the number of separate follicle cell domains with high BR expression, is determined by a single gradient of EGFR signaling. The single peak of EGFR signaling specifies the roof domains by activating both BR in a wide dorsal domain and Pointed (PNT), an ETS-domain transcription factor that represses BR, in a narrower midline region. Furthermore, we find that splitting of the EGFR signaling pattern occurs later in oogenesis and does not influence the number of domains in the BR pattern. The feedback loops mediated by *rho* and *argos* are essential for establishing the two-peaked pattern of EGFR activation, but play only a secondary role in eggshell patterning and morphogenesis.

MATERIALS AND METHODS

Fly stocks and clonal analysis

The FLP/FRT recombinant technique (Xu and Rubin, 1993) was used to generate loss-of-function clones, null clones of which are marked by the loss of a GFP marker, either cytoplasmic (*ubi-GFP*) or nuclear (*hv-GFP*). We confirmed that the $argos^{\Delta 7}$ allele, which was used for clonal analysis, does not complement either the $argos^{P1}$ (Okano et al., 1992), $argos^{257}$ (Okano et al., 1992) or $argos^{W11}$ (Freeman et al., 1992) alleles. For the complementation test, adults were examined for the appropriate dominant marker to determine whether at least one third-chromosome balancer was present. Adult flies lacking the balancer showed the characteristic eye phenotype in every case examined (see Fig. 2A-A″).

Other genotypes used in the clonal analyses include:

$argos^{-/-}$ mosaic clones. *yw hsflp*; *e22c>flp*; $argos^{\Delta 7}$ *FRT80B/ubi-GFP FRT80B* (Voas and Rebay, 2003). Clones were generated with the *e22c*-GAL4 driver and were not heat shocked.

$rho^{-/-}$ mosaic clones. *e22c>flp*; rho^{del1} *FRT80B*/*ubi-GFP FRT80B* (Bier et al., 1990) and *e22c>flp*; rho^{7M} *FRT80b*/*ubi-GFP FRT80B* (Wasserman and Freeman, 1998). We confirmed that the *rho* alleles do not complement each other by scoring adult flies.

$sty^{-/-}$ mosaic clones. *e22c>flp*; $sty^{\Delta 5}$*FRT2A/hv-GFP FRT2A* (Hacohen et al., 1998).

$kek1^{-/-}$. Two overlapping deficiencies, RA5 and RM2, completely delete *kekkon-1*. The cross *RA5/RM2* is denoted $kek1^{-/-}$ in this study (Ghiglione et al., 1999; Musacchio and Perrimon, 1996).

$kek1^{-/-}$; $sty^{-/-}$ mosaic clones. *yw hsflp122*; RA5/RM2; $sty^{\Delta 5}$*FRT2A/hv-GFP FRT2A* flies were heat shocked for 2 consecutive days and dissected and immunostained 5-10 days later, which was varied so as to obtain a range of clone sizes and frequencies.

$pnt^{-/-}$ mosaic clones. *e22c>flp*; *FRT82B* $pnt^{\Delta 88}$/*FRT82B ubi-GFP* (Morimoto et al., 1996; Scholz et al., 1993).

Ore R was used as the wild-type control in determining the size of the roof domain.

Immunostaining, microscopy and imaging analysis

Antibodies used included mouse anti-BR core (1:100, DSHB), rabbit or sheep anti-GFP (1:1000, Chemicon International and Biogenesis, respectively) and rabbit anti-dpERK (1:100, Cell Signaling). DAPI (VECTASHIELD Mounting Medium for Fluorescence with DAPI, Vector Laboratories) was used to stain for nuclei. Alexa Fluor- and Oregon Green-conjugated secondary antibodies (1:1000, Molecular Probes) were used. A standard immunostaining protocol was followed with modifications (Laplante and Nilson, 2006). For anti-dpERK stainings, ovaries were dissected and immediately placed on ice in a fixation solution (600 μl heptane, 100 μl PBST containing 8% paraformaldehyde). After dissecting several ovaries for a maximum duration of 10 minutes, the solution was diluted to ~4% paraformaldehyde. After an additional 10-minute fixation, the sample was treated with proteinase K (12.5 μg/ml, Sigma-Aldrich) for 1 minute to improve the signal-to-noise ratio. Images were acquired using the Nikon Eclipse E800 compound microscope or a Zeiss 510 confocal microscope and processed and organized with ImageJ (1997-2006, W. S. Rasband, NIH, Bethesda, MD, USA). Imaging steps were limited to the uniform subtraction of background signal and the Despeckle function provided by ImageJ. Counting of cells with high levels of BR expression was performed manually. Measurements are reported as the mean±s.e.m.

RESULTS

A single peak of MAPK signaling represses Broad in the midline

The specification of two DAs depends on the two-domain expression pattern of BR, which controls a number of genes in the future roof cells (Ward and Berg, 2005; Zartman et al., 2008). This pattern is established in a stepwise manner. BR, which is expressed in all oocyte-associated follicle cells during mid-oogenesis, becomes strongly repressed in the midline region in early stage 10B egg chambers (Fig. 1). Later, BR expression increases in the two prospective roof domains and remains stable throughout subsequent appendage morphogenesis (Dorman et al., 2004; James and Berg, 2003). Both the midline repression of BR and its upregulation in the prospective roof cells depend on the RAS/MAPK pathway, which is stimulated by activated EGFR (Atkey et al., 2006; Yakoby et al., 2008b).

Since MAPK activation is very dynamic during the time window that corresponds to the formation of the two-domain BR pattern (Kagesawa et al., 2008; Nakamura and Matsuno, 2003; Peri et al., 1999), we investigated the relative order of events in the dynamics of BR expression and MAPK phosphorylation. Using a modified immunostaining protocol (see Materials and methods), we were able to robustly obtain images of egg chambers stained simultaneously for BR and phosphorylated MAPK (dpERK; Rolled – FlyBase) in the wild-type and mutant backgrounds. The most significant finding was that the midline repression of BR occurs when MAPK is still activated in a single-peaked pattern (Fig. 1A-B‴). A detailed description of the two patterns and their interpretation in terms of previously discovered regulatory mechanisms are provided below.

The pattern of dpERK staining during stage 10A has a cusp-like shape that reflects the shape of GRK secretion from the oocyte at this stage of oogenesis (Kagesawa et al., 2008; Neuman-Silberberg and Schupbach, 1996; Pizette et al., 2009) (Fig. 1A-A‴). Remarkably, this cusp-like pattern is conserved across *Drosophila* species (Kagesawa et al., 2008). The midline repression of BR occurred during stage 10B, when the dpERK pattern still had a single peak in the midline (Fig. 1B-B‴). When the levels of BR

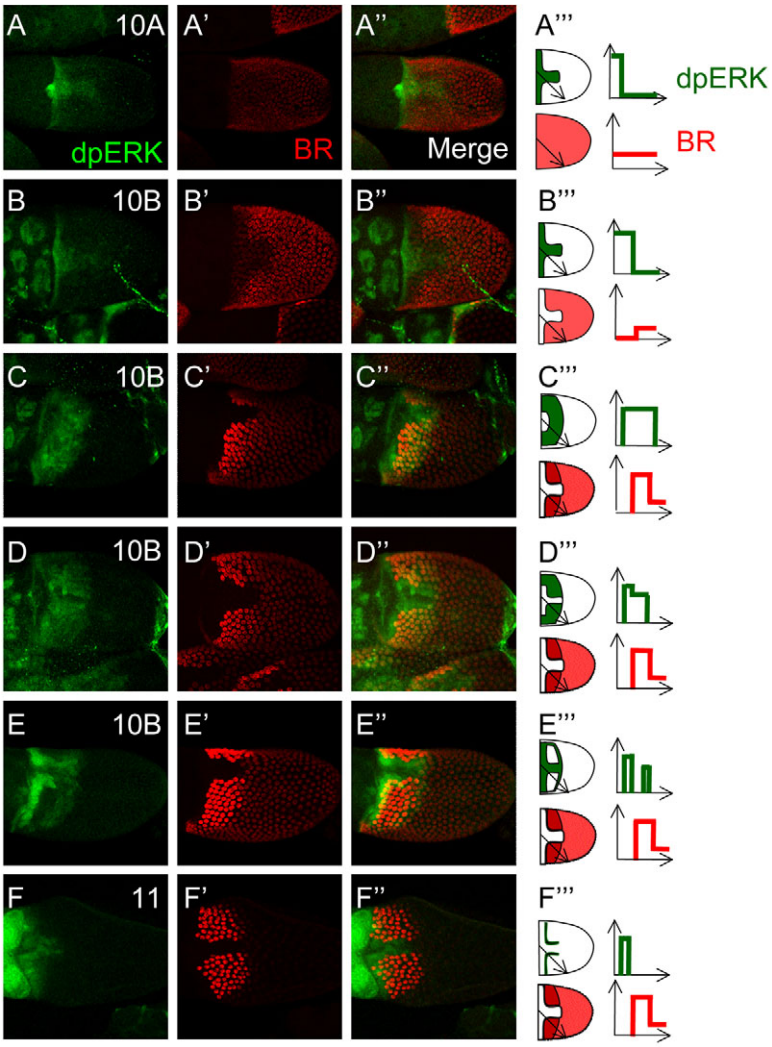

Fig. 1. Wild-type dynamics of dpERK and BR expression. Stage 10-11 *Drosophila* egg chambers stained for (A-F) phosphorylated ERK/MAPK (dpERK) or (A′-F′) BR; (A″-F″) merged channels. (A‴-F‴) Diagrams summarizing the levels of dpERK and BR at each stage at three levels of expression (low, basal or high). Cross-sections are also shown (along the arrow) of the MAPK and BR expression profiles. The BR expression profile becomes stable by mid stage 10B, and the MAPK profile is refined to the floor cells by stage 11. (**A-A‴**) High levels of dpERK are found in the dorsal midline and in an anterior band in stage 10A egg chambers. The cusp-like pattern is defined as the midline pattern. BR levels are initially uniform in the main body follicle cells that contact the oocyte. (**B-B‴**) High levels of dpERK in the midline of stage 10B egg chambers correlate with the repression of BR, which is expressed at a basal level in the posterior and ventral cells. (**C-C‴**) dpERK levels decrease in a narrow region in the dorsal anterior (arrow), but have expanded to include the roof domain as marked by BR, and in the midline space between the roof cells. (**D-D‴**) dpERK is found in the floor cells, roof cells (marked by BR) and in the cells that are found in the dorsal anterior corner. (**E-E‴**) dpERK expression increases in the floor cells and decreases in the roof cells. A posterior ring ('spectacle' pattern) surrounding the posterior boundary of BR expression also shows dpERK expression. (**F-F‴**) By stage 11, dpERK is found in the floor cells, which begin to slip under the roof cells as tube formation proceeds at stage 12. BR remains at high levels in the roof primordia.

began to rise in the future roof cells during stage 10B, MAPK activation had spread to include more of the dorsal follicle cells; the shape of the boundary of the region with high levels of dpERK had changed from cusp-like to circular (Fig. 1C-C‴). In egg chambers with this expanded pattern of MAPK signaling, the dpERK levels were downregulated in a subset of the dorsal anterior follicle cells. Note, however, that this region is significantly smaller than the separation between the two roof cell domains (Fig. 1C-E). The repression of dpERK signals in these egg chambers closely matched the dynamic pattern of *argos*, which has been reported elsewhere (Peri et al., 1999; Nakamura and Matsuno, 2003; Yakoby et al., 2008a) (see Fig. P3 in the supplementary data of Yakoby et al.). The initial region of reduced dpERK signal was limited to a small band of anterior cells (Fig. 1C-C‴). Later, the domain of downregulated MAPK signaling expanded along the dorsal midline (Fig. 1D-D‴), corresponding to a later pattern of *argos* expression.

The dpERK signal in the floor cells increases in late stage 10B egg chambers, whereas expression in the prospective roof cells decreases, forming the previously described 'spectacle' pattern (Fig. 1E-E‴) (Peri et al., 1999). At this stage, the dpERK pattern mirrors the pattern of *rho* (Peri et al., 1999), proposed to be essential for EGFR activation in late stages of oogenesis (Wasserman and Freeman, 1998). The repression of dpERK in the roof cells is consistent with the previous finding that BR represses *rho* in this region (Ward et al., 2006). Since *rho* is essential for the late phase of EGFR signaling in the follicle cells (Sapir et al., 1998; Peri et al., 1999), its repression in the roof domain is accompanied by downregulation of EGFR signaling and reduced dpERK levels. The dpERK pattern became fully split only later during stage 10B of oogenesis, after the expression of BR had already settled into a pattern with two dorsolateral domains (Fig. 1F-F‴). Thus, the two-domain nature of BR expression is established when MAPK is still activated in a single-peaked pattern.

argos splits the domain of MAPK signaling but is not essential for specifying the number of dorsal appendages

Based on the clear temporal difference in the formation of the two-domain patterns of BR and dpERK, we hypothesized that the mechanism that splits the spatial pattern of EGFR signaling is decoupled from the mechanism that generates the two-domain pattern of BR expression. To test this, we used the FLP/FRT technique (Golic and Lindquist, 1989) to generate mosaic epithelial layers with clones of $argos^{-/-}$ cells. In these experiments, we used the $argos^{\Delta 7}$ allele, which we have confirmed through complementation tests (see Materials and methods) and by reproducing the previously described eye patterning phenotype (Fig. 2A-A″).

As predicted by the Wasserman-Freeman model, we established that the removal of *argos* indeed prevents the splitting of the dpERK pattern. The first difference in dpERK patterns between wild-type egg chambers and those with dorsally located *argos*$^{-/-}$ clones was found at stage 10B, when the wild-type dpERK pattern spans the midline and the roof domains. In such *argos*$^{-/-}$ clones, the dpERK pattern did not show the characteristic downregulation in the dorsal anterior that is observed in Ore R egg chambers that have a similar dorsal anterior dpERK pattern (compare Fig. 2B-B″ with Fig. 1C-D). Downregulation of dpERK was also not detected in *argos*$^{-/-}$ clones that span the midline at a later stage, when dpERK levels are attenuated in the roof cells (compare Fig. 2C-C″ with Fig. 1E-E″). Even in stage 11/12 egg chambers, ectopic levels of dpERK were found in the midline for *argos*$^{-/-}$ clones [compare Fig. 2D-D″ (stage 11/12) with Fig. 1E-E″]. Importantly, this loss of peak-splitting of the dpERK gradient did not prevent the formation of a fully developed two-domain pattern of BR.

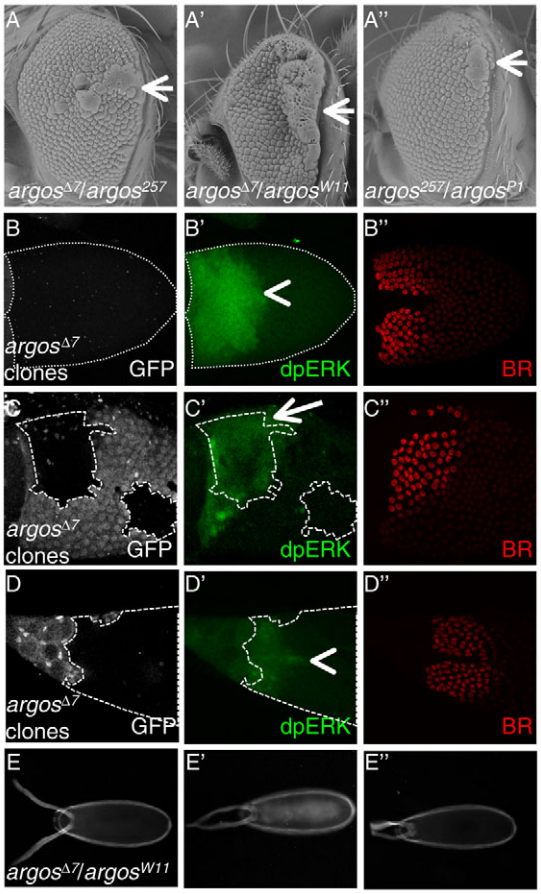

Fig. 2. The effects of *argos* on MAPK signaling and eggshell patterning. (**A-A″**) The *argos*$^{\Delta7}$ *FRT80B* line, which was used for mosaic analysis, does not complement other mutant alleles of *argos* as demonstrated by the eye blister phenotype for *argos*$^{\Delta7}$ *FRT80B*/*argos*^{257} (A), *argos*$^{\Delta7}$ *FRT80B*/*argos*^{W11} (A′) and *argos*^{257}/*argos*^{P1} (A″) flies. The arrow denotes the midline. (**B-B″**) An *argos*$^{\Delta7}$ clone that spans all of the main body follicle cells, marked by loss of GFP (B), shows a single peak of dpERK staining (B′) at a time when comparable wild-type egg chambers show a loss of dpERK in a narrow midline region (see Fig. 1C-E). The midline is marked by an arrow. (**C-C″**) In another large *argos*$^{\Delta7}$ clone spanning the dorsal domain, dpERK staining remains at high levels in the dorsal anterior (arrow, C′), in contrast to the pattern found in wild-type egg chambers (see Fig. 1E-E″), when the spectacle pattern is present. (**D-D″**) In a stage 12 egg *argos*$^{\Delta7}$ clone, a single row of elevated dpERK is still seen (arrow, D′), which is where the *argos* transcript is normally expressed (data not shown). (**E-E″**) Examples of *argos* mutant eggs showing both wild-type and aberrant dorsal appendage (DA) morphologies. The majority of eggs examined showed wild-type DA morphology (E). Other phenotypes with low penetrance included reduced inter-appendage distance (E′) and appendages that were shorter (E″). Shown are eggshells laid by *argos*$^{\Delta7}$/*argos*^{W11} females.

Thus, the two-domain pattern of BR can be formed by a single gradient of MAPK activation. Since the two-domain pattern of BR is essential for the formation of two DAs, this conclusion contradicts the current model, according to which the splitting of the spatial domain of EGFR signaling is essential for proper eggshell patterning (Wasserman and Freeman, 1998). Furthermore, the number of BR-expressing cells that define the roof domain in large *argos*$^{-/-}$ clones covering the dorsal follicle cells was the same as in wild-type (Ore R) egg chambers. The number of BR-expressing cells in egg chambers with large *argos*$^{\Delta7}$ clones spanning the dorsal half of the egg chamber was 53±1 (s.e.m.) (n=19), whereas Ore R egg chambers had 51±1 (n=53). This difference is not statistically significant (P=0.18).

All of the examined eggshells that were derived from females with *argos*$^{\Delta7}$ mosaic egg chambers had two DAs (n=1046 eggs), with only a small fraction showing morphogenesis defects that ranged from a loss of DAs, shorter DAs, and DAs with reduced inter-appendage distances [91/1046 (11%) of eggs examined]. A small fraction of eggshells that were *argos* hypomorphs also showed a reduction in inter-appendage distance: 5% (55/1191) of *argos*$^{\Delta7}$/*argos*^{W11} and 3% (33/1105) of *argos*^{257}/*argos*^{P1} eggs differed from the wild-type phenotype, but no fused appendages were observed (Fig. 2E-E″). Therefore, Argos is involved in splitting the pattern of MAPK signaling and might also play a role in the process of DA morphogenesis, but does not determine the number of DAs.

rho is essential for the late phase of EGFR signaling but not for specifying the number of dorsal appendages

One of the key components of the patterning model proposed by Wasserman and Freeman is *rho*, which encodes an intracellular protease essential for the processing and secretion of Spitz, a ubiquitously expressed EGFR ligand (Lee et al., 2001; Schweitzer et al., 1995; Tsruya et al., 2007; Urban et al., 2001). *rho* is induced by GRK and exhibits a very dynamic expression pattern in the follicle cells (Peri et al., 1999; Ruohola-Baker et al., 1993). The onset of *rho* expression, and consequently EGFR activation by Spitz, follows the final phase of GRK signaling during stage 10B. Initially, *rho* is expressed in a large dorsal domain, but is subsequently downregulated in the midline and roof domains to stabilize in a pattern of two L-shaped domains that mark the floor cells (Peri et al., 1999; Ruohola-Baker et al., 1993). Based on the similarities in the spatiotemporal patterns of *rho* expression and MAPK phosphorylation, *rho* was proposed to amplify and expand the spatial domain of EGFR activation by GRK (Peri et al., 1999). The two-domain pattern of *rho* accounts for the two peaks of EGFR signaling (Wasserman and Freeman, 1998).

To directly explore the patterning function of *rho*, we examined MAPK phosphorylation and BR expression in egg chambers with marked clones of *rho*$^{-/-}$ cells. In these experiments, we used the *rho*^{7M} allele (Wasserman and Freeman, 1998), which we confirmed does not complement a second allele, *rho*^{del1} (Bier et al., 1990) (see Materials and methods). We found that the early pattern of dpERK

was unaffected in early stage 10 egg chambers with large or complete clones of rho^{7M} cells (Fig. 3A-A″), as expected given that the early phase of MAPK activation does not depend on the positive feedback provided by Rhomboid and Spitz.

The later phase of MAPK signaling, however, was completely abolished in clones of rho^{7M} cells. These observations are consistent with the Wasserman-Freeman model and with our previous computational studies, according to which *rho* is essential for the late phase of MAPK signaling in the follicular epithelium (Shvartsman et al., 2002; Wasserman and Freeman, 1998). The effect of *rho* on MAPK appears to be short range. For example, when a clone of $rho^{-/-}$ cells partially overlapped with the endogenous late pattern of *rho*, MAPK signaling was affected only in the mutant cells (Fig. 3B-B″). Apparently, Spitz secreted from the wild-type cells was not sufficient to induce MAPK signaling in the mutant cells located several cell diameters away.

These observations suggest that the positive-feedback loop formed by Rhomboid, Spitz and EGFR operates in a regime whereby a secreted ligand is captured and degraded within close proximity to its release point (1-2 cell diameters) (Pribyl et al., 2003a). Thus, the length scale of autocrine Spitz is significantly shorter than the length scale of GRK, which acts as a long-range paracrine signal in patterning of the follicle cells (Chang et al., 2008; Goentoro et al., 2006; Pai et al., 2000). This conclusion is consistent with results from previous experimental studies of the relative effects of GRK and Spitz on *pipe*, a gene that is expressed in the ventral follicle cells (Peri et al., 2002), and with independent estimates of the length scale of Spitz in the eye imaginal disk and embryonic ventral ectoderm (Freeman, 1997; Reeves et al., 2005).

Despite the fact that *rho* clearly affects the dynamics of MAPK signaling, it does not control the expression of BR, as both the early and late patterns of BR are normal in egg chambers with clones of rho^{7M} cells (Fig. 3) and rho^{del1} (data not shown). Furthermore, we examined eggs with unmarked mosaic clones of rho^{7M} and never observed fused appendages; only a low percentage (4%, 51/1366) of eggshells showed defects in DA size or inter-appendage distance. Similarly, we never observed fused appendages in rho^{del1} mosaic eggs, and only a low percentage of eggshells showed defects in the size or spacing of the appendages (15%, 105/713). Taken together, these data strongly suggest that the early phase of BR repression is mainly due to a single-peaked gradient of EGFR activation by GRK. Thus, the Rhomboid/Spitz/Argos module dictates the late pattern of EGFR signaling and affects morphogenesis at a low rate of penetrance, but does not regulate the number of DAs.

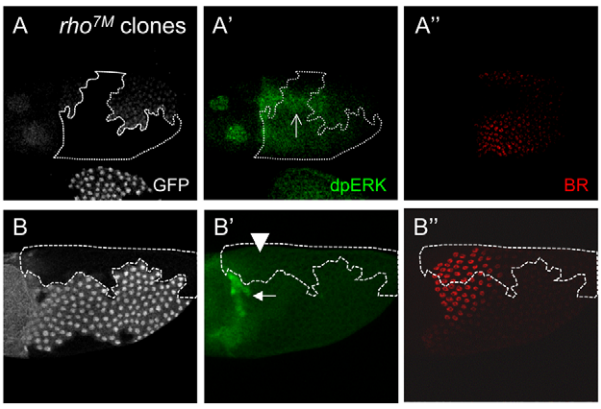

Fig. 3. The effects of *rho* on MAPK signaling and BR expression. (**A-A″**) In early stage 10B *Drosophila* egg chambers, rho^{7M} clones show no effect on dpERK levels in the midline (arrow, A′) or on repression of BR in the midline (A″). (**B-B″**) *rho* is locally required for MAPK activity in the floor cells (B′, arrowhead), as shown in a clone that covers the dorsal edge of the floor domain (B,B′, arrow). The BR domain is unaffected (B″).

kek1 and *sty* regulate the size and position, but not the number, of BR expression domains

In addition to *argos*, which inhibits EGFR activation extracellularly by ligand sequestration, EGFR also induces two intracellular inhibitors of EGFR signaling in the follicular epithelium: *kek1* and *sty*. KEK1 is a transmembrane protein that inhibits signaling by direct interaction with EGFR (Ghiglione et al., 1999). STY is a highly conserved intracellular protein that inhibits signal transduction downstream of activated receptor tyrosine kinases, including EGFR (Casci and Freeman, 1999; Hacohen et al., 1998; Reich et al., 1999). The removal of either *kek1* or *sty* leads to dorsalized eggshells, but the precise patterning function of these inhibitors in the follicular epithelium has remained unclear.

Removal of *kek1* leads to eggshells with thin and widely spaced appendages (Ghiglione et al., 1999) (Fig. 4A). Based on this, we expected that in the $kek1^{-/-}$ background the two domains of BR should be further apart and contain a reduced number of cells. Indeed, we found that the size of the prospective roof domains was significantly reduced in the $kek1^{-/-}$ egg chambers as compared with the wild type (44±1 cells, n=18, $P=1\times10^{-4}$). Importantly, we found that removal of *kek1* does not affect the dynamics of BR expression. Similar to in the wild-type background, the two-domain pattern of BR was established in a characteristic sequence of midline repression and upregulation in the prospective roof cells (Fig. 4A′,A″). At the same time, the size of the midline region that corresponds to the early repression of BR clearly increased (Fig. 4A′). Thus, the eggshell phenotype of *kek1* can be traced back to the early (repressive) phase of formation of the roof cell domain.

We next compared the reported eggshell phenotype of *sty* with the pattern of BR expression in *sty* mosaic egg chambers. In agreement with previous reports (Reich et al., 1999), we identified a low frequency of eggshells with multiple appendages (Fig. 4B). Based on experiments with marked mosaic egg chambers, we established that the effect of small clones of $sty^{\Delta5}$ cells is position dependent: clones in the midline-most region had no effect on BR, indicating that *sty* is not essential for BR repression in this region (Fig. 4B,B″, arrowhead). However, small clones located in the middle of the roof domain led to repression of BR (Fig. 4B,B″, arrow). Clones that spanned the boundary of the BR domain generated an additional boundary between the normal roof cells and the dorsal midline. This could account for the occasional formation of extra DAs (Reich et al., 1999) (Fig. 4B). The most common eggshell phenotype was characterized by thinner and widely spaced DAs (Fig. 4C). Complete removal of *sty* had an effect on BR that was qualitatively similar, yet stronger, than that observed upon removal of *kek1*: BR was still expressed in two domains, but their size was greatly reduced (23±1 cells, n=22, $P=4\times10^{-28}$). Similar to *kek1* egg chambers, the increased separation of the BR patches in the final two-domain pattern in *sty* egg chambers could be attributed to the early phase of BR dynamics, when BR is repressed in the dorsal midline (Fig. 4C′,C″).

Removal of *sty* in $kek1^{-/-}$ egg chambers gave an even stronger phenotype than *kek1* alone: $kek1^{-/-}$ egg chambers with small clones of $sty^{-/-}$ cells showed an increase in the size of the midline (Fig.

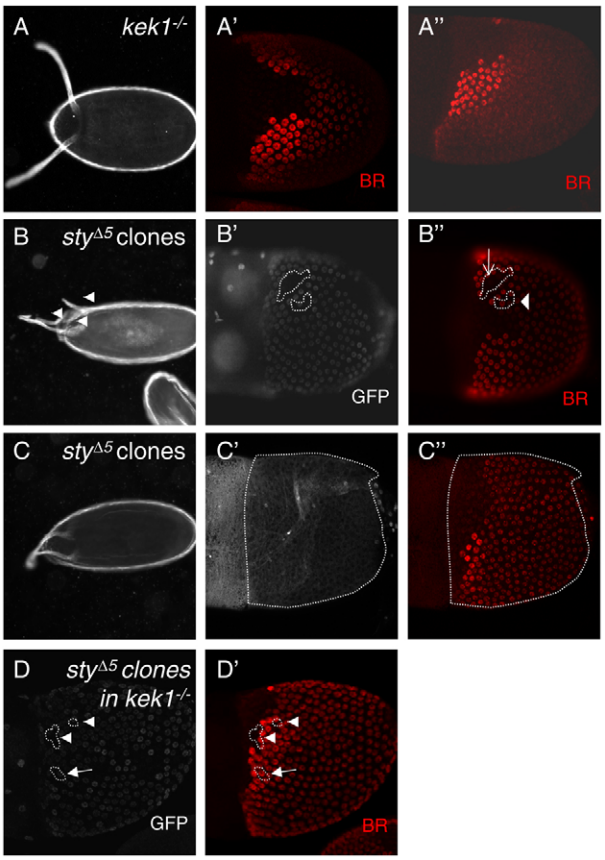

Fig. 4. *kek1* and *sty* modulate the size of the roof domain and inter-appendage distances. (**A-A″**) *kek1*$^{-/-}$ *Drosophila* egg chambers have DAs that are further from each other (A) than in wild type, consistent with the increased size of the midline domain (~4-5 cells), which is marked by low levels of BR (A′), and with the reduced size of the roof domain (A″, stage 10B; see also Fig. 6C). (**B-B″**) A small percentage of eggshells exhibits multiple, ectopic appendages (B, arrowheads), consistent with the creation of ectopic boundaries of BR with the midline (B′,B″, arrow). BR remains repressed in small clones located in the midline (B′,B″, arrowhead). (**C-C″**) The most common eggshell phenotype in unmarked *sty*$^{\Delta 5}$ clones exhibits thinner DAs that are located more laterally (C). This phenotype is consistent with a large expansion in the midline (~10-11 cells; see also Fig. 5E) and a significantly reduced BR patch size (C′,C″). (**D**,**D′**) *kek1*$^{-/-}$ egg chambers with small *sty*$^{-/-}$ clones show a superposition of patterning phenotypes: the dorsal midline has increased, similar to the *kek1*$^{-/-}$ pattern, and *sty*$^{-/-}$ clones located within the dorsal half of the BR patch lead to a loss of elevated BR expression (arrowheads). *sty*$^{-/-}$ clones located in the ventral side of the roof domain are unaffected (arrow).

4D,D′), consistent with the patterning effect of *kek1*$^{-/-}$ egg chambers. Furthermore, small clones of *sty*$^{-/-}$ that were located within the dorsal half of the roof domain lost the elevated expression of BR (Fig. 4D,D′, arrowheads), but clones in the ventral-most part of the patch did not (Fig. 4D,D′, arrow). Thus, *kek1* and *sty* control the size and location of the prospective roof cell domains, but are not essential for defining their number. In this respect, they are similar to Argos, which is likewise not essential for defining the number of domains in the BR expression pattern.

Finally, we tested whether KEK1 and STY, similar to Argos, are involved in defining the split pattern of MAPK signaling. Based on the simultaneous detection of dpERK and BR in *kek1*$^{-/-}$ egg chambers, we found that *kek1* does not affect the pattern of MAPK signaling (Fig. 5A-B″), but does increase the separation between the two domains of MAPK signaling (Fig. 5B-B″). Removal of *sty* delayed the splitting of the dpERK pattern: the pattern of dpERK was clearly single-peaked even after stage 10B of oogenesis, when it is fully split in the wild type (Fig. 5C-C″, compare with Fig. 1C-E; as discussed above, the relative staging of the two egg chambers is based on the fact that when the domain of dpERK is expanded to include the roof cells, dpERK is strongly downregulated in the midline as well). In contrast to the response of BR expression, the increase in dpERK staining did not appear to be cell-autonomous in small *sty*$^{-/-}$ clones (Fig. 5D-D″). However, the BR domains were already fully specified by this stage. In later stage 10B/11 egg chambers, dpERK levels were still detected above background in the midline, but the highest levels of dpERK were specified in the prospective floor cells (Fig. 5E-E″). Thus, both Argos and STY affect the late phase of MAPK signaling.

Negative feedback tunes the output of an incoherent feedforward loop activated by GRK

Our observations at this point can be summarized as follows. First, the number of domains in the expression pattern of BR is specified before the pattern of MAPK signaling is split along the dorsal midline (Fig. 1). Second, removal of any one of the three EGFR inhibitors does not affect the number of BR domains, nor does it lead to egg chambers with fused appendages (Figs 2, 4 and 5). Third, Argos and Rhomboid, which are essential for defining the two-peaked pattern of EGFR activation, have only a minor effect on the shape of the roof domain and on eggshell morphology (Figs 2 and 3). Fourth, the effects of *kek1* and *sty* are manifested during the initial stage of specification of the BR domain during early stage 10B, which corresponds to the single gradient of EGFR activation (Figs 4 and 5). At the level of follicle cell patterning, removal of either *kek1* or *sty* causes the domain of high EGFR activity to expand laterally, leading to an increased separation between the two domains of BR expression. On the basis of these observations, we argue that the split pattern of MAPK signaling is not essential for specifying the two domains of BR expression and DA morphogenesis.

Instead, our observations are consistent with the previously proposed mechanism whereby the two-domain pattern of BR is established by an incoherent feedforward loop, i.e. a network in which an input activates both a target gene and its repressor (Kaplan et al., 2008; Lembong et al., 2009; Yakoby et al., 2008b). In this case, the input is provided by the single-peaked pattern of EGFR activation by GRK, the target gene is *br*, and its repression is mediated by PNT, an ETS-domain transcription factor (Boisclair Lachance et al., 2009; Lembong et al., 2009; Morimoto et al., 1996; Yamada et al., 2003). A repressive role for PNT is supported by the fact that eggshells derived from egg chambers with clones of *pnt*$^{-/-}$ (*pnt*$^{\Delta 88}$) cells have a single DA, and the fact that the midline *pnt*$^{-/-}$ clones led to cell-autonomous ectopic expression of BR (Fig. 6A-A″). It is unclear whether repression of BR by PNT is direct or indirect; we note, however, that repression mediated by ETS-domain transcription factors has been reported in other developmental contexts as well (Mao et al., 2009; Zhang et al., 2009).

In an updated version of this mechanism, the two-domain output of the feedforward loop is quantitatively controlled by the intracellular feedbacks provided by *kek1* and *sty* (Fig. 6B). Following induction in the dorsal midline region in response to the earlier phase of EGFR signaling, *kek1* and *sty* reduce the level of EGFR activation in the midline and in this way reduce the domain of the repressive action of PNT (Fig. 6B′,B″). Removal of either one

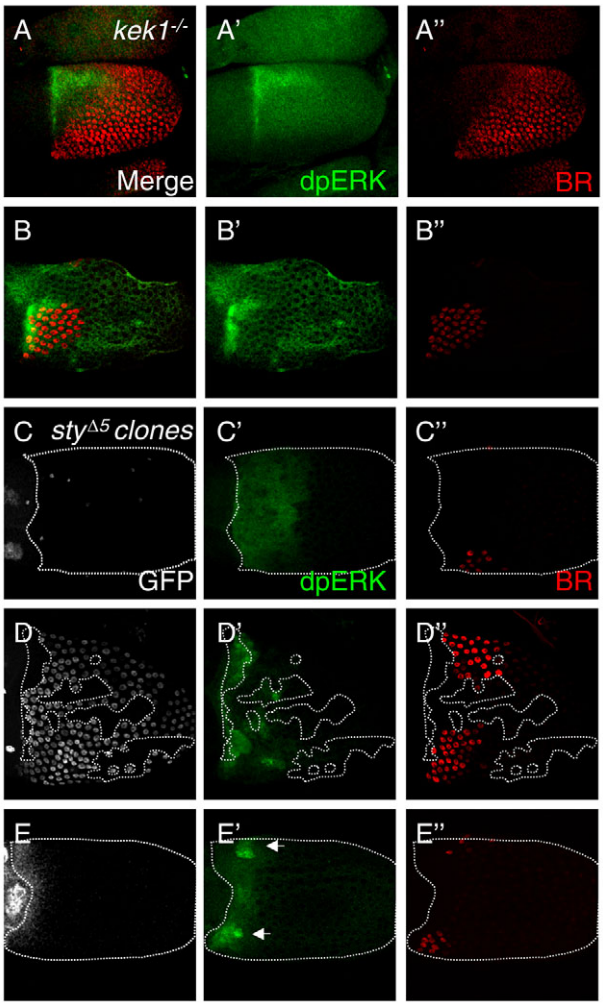

Fig. 5. *kekkon-1* and *sprouty* are not required for splitting the peak of MAPK activity. (**A-A″**) In stage 10B *kek1*$^{-/-}$ *Drosophila* egg chambers, a single gradient of dpERK is detected during specification of the midline (loss of BR) and of the two separated BR domains, similar to as in Ore R. (**B-B″**) The split domains of activated MAPK (green) are detected in older *kek1*$^{-/-}$ egg chambers. The only difference from the wild type is the increased separation between the two domains of DA primordia. (**C-C″**) In large *sty*$^{-/-}$ clones, a single large domain of dpERK spans the midline. (**D-D″**) The increase in dpERK in small clones located in the midline is not fully cell-autonomous (D′), in contrast to the effect of *sty* clones on BR. Note that this effect is evident after the two BR domains have already been specified. In some cases, the level of BR is variable at stage 10B in *sty*$^{-/-}$ clones (D″). (**E-E″**) Although elevated dpERK staining is detected in large *sty*$^{-/-}$ clones in late stage 10B/11 egg chambers, the highest levels of dpERK (E′, arrows) are detected in two sets of floor cells, anterior to the roof domain, as marked by BR (E″).

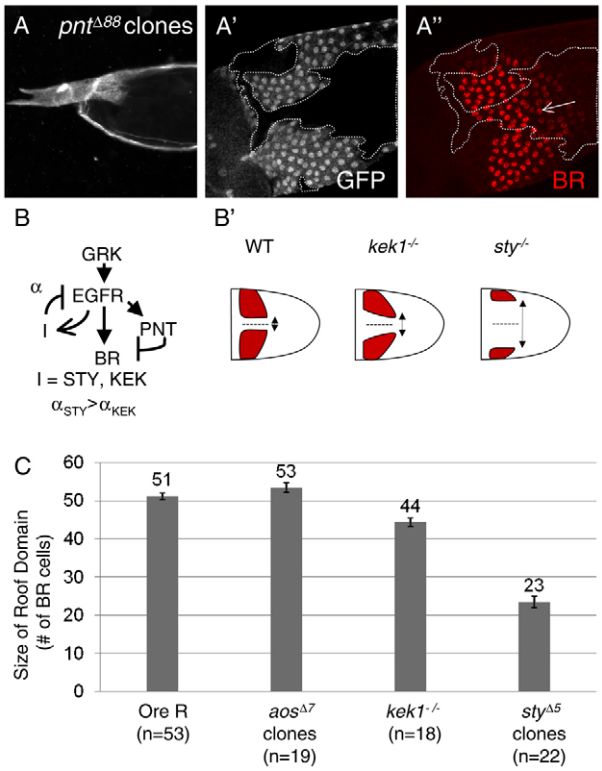

Fig. 6. Negative feedbacks modulate the output of an incoherent feedforward loop. (**A-A″**) *Drosophila pnt*$^{\Delta 88}$ mosaic eggs show a single DA that includes the dorsal midline, which is consistent with elevated BR expression in clones that span the midline (A,A′). Ectopic BR is found in the clone spanning the midline. The follicle cells that form the anterior-most two rows over the oocyte show repression of BR that is independent of PNT repression. The arrow indicates the approximate location of the midline. (**B**,**B′**) Model for specifying the two domains of roof cells. BR is activated by GRK-induced EGFR signaling during stage 10B in a wide domain of dorsal follicle cells. High levels of EGFR signaling lead to repression of BR, which is mediated by PNT. Cell-autonomous inhibitory feedback by KEK1 and STY modulates the location of the dorsal domain of BR. The strength of inhibitory feedback, α, is stronger for STY than for KEK1. (B′) As a result, the size of the midline increases at the expense of the roof domain in egg chambers mutant for either *kek1* or *sty*. (**C**) Quantification of the effect of each inhibitor on the size of the BR patch. Argos has a negligible effect, whereas *kek1*$^{-/-}$ and *sty*$^{-/-}$ egg chambers have a reduced number of BR cells in each DA primordial (see text for *P*-values).

of the inhibitors leads to an increase of EGFR signaling in the midline and increases the separation between the two BR domains. As a result, the number of cells with elevated levels of BR decreases. This model predicts that a reduction in feedback strength reduces the size of the BR patches by shifting their dorsal boundary, which is consistent with our analysis of the number of BR-expressing cells in the wild-type and mutant backgrounds (Fig. 6B′,C). Thus, in our model, one function of the negative-feedback loops provided by *kek1* and *sty* is to indirectly control the levels and domain of expression and action of PNT.

DISCUSSION

The morphogenesis of structures with repeated functional units, such as body segments and appendages, depends on multi-domain patterns of cell signaling and gene expression. Such patterns can form either by inductive and cell-autonomous mechanisms or they rely on cell-cell interactions and feedback. As an example of a purely inductive mechanism, the two symmetrical gene expression domains in the prospective neuroectoderm in the early *Drosophila* embryo are formed by a single-peaked Dorsal morphogen gradient that is interpreted by a cell-autonomous incoherent feedforward loop (Zinzen et al., 2006). By contrast, the formation of quasi-periodic two-dimensional transcriptional patterns that prefigure the formation of hair follicles and feathers depends on non-cell-autonomous mechanisms (Sick et al., 2006).

We found that both types of mechanism operate side-by-side during the patterning of the follicular epithelium. A largely cell-autonomous network, based on an incoherent feedforward loop, defines the two-domain pattern of BR, a transcription factor essential for the formation of the two eggshell appendages (Fig. 6). This patterning event depends on a single-peaked gradient of EGFR activation in the follicular epithelium. During later stages of follicle cell patterning, when the long-range GRK signal is replaced by the short-range Spitz, this gradient is split under the action of the previously characterized network of feedback loops (Wasserman and Freeman, 1998).

In contrast to the currently accepted autocrine feedback model of eggshell patterning (Wasserman and Freeman, 1998), we argue that the formation of the split pattern of MAPK signaling is not essential for specifying the two DAs. This is based on the fact that splitting of MAPK signaling occurs later than the specification of the two domains of BR, and that the BR pattern is specified correctly in *argos*$^{-/-}$ egg chambers that exhibit a single peak of MAPK signaling. Thus, the negative feedback by Argos splits the spatial pattern of EGFR activation, but does not dictate the number of DAs. We speculate that the partially penetrant eggshell phenotype of *argos* can be attributed to quantitative changes in the shape of the BR domain or in the regulation of appendage morphogenesis. This hypothesis could be tested by live imaging of DA morphogenesis in *argos* mutants.

The patterning effects of *kek1* and *sty* can be interpreted within the framework of a model in which the number of domains in the expression pattern of BR is established by an incoherent feedforward loop; the intracellular inhibitors of EGFR control the size and location of these domains. We emphasize that this model accounts only for the dorsoventral character of the BR pattern and for the early phase of BR expression, when it is controlled by a single gradient of EGFR activation. Explaining the anteroposterior character of BR expression requires extending this model to include interactions with the DPP pathway, which acts to control the anterior boundary of the roof domain as well as the temporal amplitude of *br* transcription (Shravage et al., 2007; Yakoby et al., 2008b). Description of the late, split pattern of MAPK signaling requires explicit modeling of the positive feedback through Rhomboid and Spitz and of the inhibitory action of Argos and STY (Pribyl et al., 2003b; Shvartsman et al., 2002). An integrated dynamic description of eggshell patterning could be based on existing mathematical models of EGFR and DPP signaling in the follicular epithelium (Lembong et al., 2008; Lembong et al., 2009).

The flexibility of a patterning system in which an incoherent feedforward loop is regulated by multiple negative-feedback loops, each with a different expression threshold and feedback strength, could potentially account for the diverse eggshell morphologies in other species of *Drosophila*. The changes that have been observed in the spatial pattern of BR in other species have noticeable parallels with the effects that EGFR inhibitors have on the patterning of BR in *D. melanogaster*. For example, the spacing between the two BR domains is also affected in other species, such as *D. melanica* (N. Yakoby, personal communication), and this could correspond to changes in the inhibitory feedback mediated by either KEK1 or STY, or in the shape and strength of GRK secretion from the oocyte. Additionally, the slope of the dorsal boundary of the early BR expression pattern with respect to the dorsal midline varies in other species, such as *D. virilis* (James and Berg, 2003), which is reminiscent of the effect that *kek1* has on patterning the BR patches in *D. melanogaster*. In the future, it will be interesting to compare the relative effects of inhibitory feedback in these species as a further test of our proposed model of BR patterning.

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