THE GLYPICAN DALLY SHAPES FOLLICLE CELL PATTERNING BY REGULATING THE EPIDERMAL GROWTH FACTOR RECEPTOR LIGAND GURKEN

BY

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ABSTRACT OF THE THESIS

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Heparan sulfate proteoglycans (HSPGs) have been shown to interact with morphogens of many signaling pathway. During *Drosophila* oogenesis, the major contributors to tissue patterning are the bone morphogenic protein (BMP) and epidermal growth factor receptor (EGFR) signaling pathways. It was previously shown that BMP signaling is regulated by the HSPG, *dally (division a*bnormally delayed), in the wing, and also in patterning of the follicle cells (FCs). The EGFR pathway is responsible for axis determination as well as follicle cell pattering. Using genetic perturbation, we demonstrate that Dally regulates the distribution of EGFR signaling through the restriction of the TGFα-like ligand Gurken (GRK). When *dally is* perturbed by uniform overexpression or depletion in the FCs, the GRK gradient is either narrowed, or widened, respectively. In these backgrounds, changes in EGFR activation, measured by dpERK, are consistent with the shapes of GRK patterning. These effects on EGFR activation lead to corresponding results on follicle cell pattering where a decrease in midline clearing of BR in overexpression of *dally,* corresponding to a reduced gap between the dorsal

appendages (DAs). Expressing a mutant form of Dally, lacking an anchor to the membrane, perturbed the GRK gradient, leading to tissue patterning and eggshell morphology defects. Based upon these results, we propose that Dally is required for the formation of the GRK gradient for optimal EGFR signaling activation.

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Introduction

Development from a single fertilized egg to the trillions of cells that comprise a human is a highly complex process. One of the processes involved in development is tissue patterning, which occurs through signaling molecules, called morphogens. Morphogens are chemicals that are secreted from a localized source that forms a chemical gradient from high to low the farther it travels from its source. In response to a morphogen gradient, the undifferentiated cells become non-uniform patterned tissues (Fig. 1A) (Turing, 1952). An established model system to study tissue patterning is *D*rosophila oogenesis.

Drosophila oogenesis

During oogenesis, egg chambers go through a process of 14 morphological stages, eventually forming the eggshell (Fig. 2A) (Spradling, 1993). Around half way through oogenesis, the egg chamber consists of an of oocyte, which after fertilization, will develop into the embryo, the oocyte is surrounded by a mono-layer of epithelial cells, called follicle cells (FCs), and nurse cells (NCs), which are responsible for nourishing the developing oocyte with protein and RNAs (Fig. 2B) (Spradling, 1993). At the end of oogenesis, the FCs secrete proteins that forms the 3 dimensional eggshell structure. This eggshell will house the developing embryo, and is comprised of dorsal appendages (DAs), which allow for gas exchange for the developing embryo, the

micropyle (M), which gives access to sperm for fertilization, and the operculum, which acts as a hatch for the larva to escape (Fig. 2C) (Berg, 2005; Shravage *et al*., 2007).

Tissue patterning in oogenesis

The formation of the eggshell structures is determined though tissue patterning. A well-studied example of this process is formation the dorsal appendages, in which, two populations of cells, the transcription factor, Broad (BR)-expressed in two patches, forms the upper part of the DAs, and the protease, *rhomboid* (Rho), expressed in two "L" stripes, forms the bottom part of the DAs (Fig. 3) (Berg, 2005; Shravage *et al*., 2007; Tzolovsky et al., 1999; Ruohola-Baker *et al*.,1993; Deng and Bownes, 1997). The domain between the two BR patches is a called the dorsal midline, which will develop into the operculum (Fig. 3A) (Shravage *et al*., 2007).

Intercellular signaling pathways in oogenesis

During oogenesis, the FCs receive input from several signaling pathways, including the bone morphogenetic protein (BMP) (Twombly *et al*., 1996; Dobens and Raftery, 2000; Parker *et al*., 2004; Massague and Gomis, 2006), the epidermal growth factor receptor (EGFR) (Neuman-Silberberg and Schupbach, 1994), among others. The BMP ligand is Decapentaplegic (DPP), a homolog of the human BMP2/4 and is part of the TGFβ family of signaling molecules (Fig.

4A) (Parker *et al*., 2004; Massague and Gomis, 2006). Dpp is secreted from the anterior most follicle cells, and creates an anterior-posterior gradient (Fig. 4B), where it binds to a type I, and type II BMP patterns. (Twombly, *et al*., 1996). Initially, the BMP receptor, *tkv,* is expressed uniformly throughout the follicle cells and interacts with the anterior follicle cells through phosphorylation of the intercellular mediator mothers against Dpp (MAD), to become P-Mad, where it is translocated into the nucleus with Medea (Med), and acts as a transcription factor (Fig. 4C) (Baker and Harland, 1997; Wu and Hill, 2009).

The epidermal growth factor receptor (EGFR) pathway is involved in axes formation and tissue development (Neuman-Silberg and Schüpbach, 1994). The *Drosophila* gene (*torpedo/egfr)* encodes the homolog of the vertebrate EGF receptor (Livneh et al, 1985; Wadsworth et al, 1985; Price *et al*., 1989). The EGFR pathway determines both the anterior/posterior (AP) and dorsal/ventral (DV) axis of the egg, and embryo (Queenan *et al*.,1997). Gurken (GRK), a TGFα-like ligand, is the primary molecule acting during oogenesis (Fig. 5). Prior to stage 8, GRK induces posterior follicle cell fates, thus establishing the AP axis, at later stages, when the oocyte nucleus moves to the anterior-dorsal corner of the oocyte, GRK mRNA and protein become asymmetrically localized and consequently determine the DV axis (Fig. 5) (Neuman-Silberberg and Schüpbach, 1994).

Role of glypicans in cell signaling

Morphogens have been shown to interact with the extracellular molecules. Heparan sulfate proteoglycans (HSPGs) (Lin, 2004). HSPGs regulate signal transduction by sequestering molecules, and have been shown to have an effect on the development in *Drosophila,* (Bellaiche *et al.,* 1998; Binari *et al*.,1997); Hacker *et al*., 1997; Haerry *et al*., 1997) mice, *(*Bullock *et a*l., 1998*),* and humans (Lind et al., 1998; McCormick *et al*., 1998). HSPGs are extracellular molecules (ECM) that comprise a core protein to which heparan sulfate (HS) glycosaminoglycan (GAG) chain are attached, and are anchored to the plasma membrane by a glycosylphosphatidyl inosoitol (GPI) linkage (Fig. 6). The *Drosophila* genome encodes four HSPG homolog's, two of them, glypicans, Division abnormally delayed (Dally) and Dally-like protein (Dlp), have been shown to interact with ligands from the Wingless, Hedgehog, and BMP signaling pathways during development (Han et al,. 2004; Lin 2004).

Dally in oogenesis

EGFR and BMP signaling pathways regulate *Drosophila* eggshell patterning. In oogenesis, BMP signaling was shown to be a downstream regulator of *dally* (Lemon and Yakoby, unpublished). It was also found that in depletion of *dally* in the follicle cells, ectopic P-Mad was found in the posterior end which suggests a reduction in EGFR signaling (Fig. 7B, C). This is consistent with results seen in GRK null flies (Fig. 7 A) (Lemon and Yakoby, unpublished; Peri and Roth 2000; Yakoby unpublished). Here, we propose that Dally regulates EGFR signaling during oogenesis.

Our results demonstrate that the glypican *dally* regulates the EGFR signaling pathway during oogenesis. Using genetic perturbations to ectopically expres*s dally* in the follicle cells is that in over expression of *dally* we see a narrower, and more restricted GRK gradient, which resulted in a restricted dpERK staining as compared to wild-type GRK. And when *dally* was depleted in the follicle cell, the GRK gradient was more diffuse, which showed a more diffuse dpERK staining. Over expression of *dally* resulted in a reduced clearing of BR in the dorsal midline, which corresponded to ventralized eggshells. Expressing a Dally protein lacking attachment to the plasma membrane resulted in drastic reduction in the GRK gradient as well as defects in tissue patterning and eggshell morphology.

Materials and Methods

Flies:

*D*rosophila *melanogaster* stocks include wild-type (OreR), UASDally (strong) (H. Nakato), UAS-shDally (Hudson and Cooley, Transgenic RNAi Project), E4-GAL4, GR1-GAL4 (Duffy, 2002, Qeenan et al). UAS-GFPDallyΔGPI (Susan Eaton). All flies were maintained at 23C on standard high-agar cornmeal media, activated yeast was added to fly vials 18-30 hours prior to dissection and continuously during egg collection.

Genetic tools:

The GAL4/UAS allowed for targeted misexpression of *dally* constructs. By crossing a fly which produces the yeast transcription factor GAL4 in a specific tissue and time, with a fly which contains a gene of interest with an upstream activating sequence (UAS), *dally* was depleted or overexpressed (Muqit and Feany, 2002)

Immunohistochemistry:

Immunoassay protocol was done as previously described (Pacquelet and Rorth, 2005). Ovaries were dissected in cold Grace's medium for ≤15 minutes and fixed with 4% paraformaldehyde, heptane, and PBS. Primary antibodies used were: mouse anti-Broad core (25e9.D7; 1:100, DSHB), rabbit antiphosphorylated-Smad1/5/8 (1:3500), Secondary antibodies used were: Alexa Fluor-488nm-conjugated goat anti-mouse, Alexa Fluor-568nm goat anti rabbit, Alexa Fluor-488nm donkey anti-sheep (all 1:1250), and DAPI (1:10,000). *D. melanogaster* mouse anti-Gurken (1D12, Developmental Studies Hybridoma Bank – DSHB, IA) was used 1:10. Ovaries for dpERK staining were dissected in ice cold graces medium during dissection. Due to the instability of dpERK, every pair of ovaries were immediately fixed in 80ul of PFA, 600ul of Heptane, and 120ul of 0.2% PBS Triton. After 10 minutes of dissection, samples were fixed for 20 minutes with a fresh fix solution. Fixed tissue was incubated for 1 min with protease K (1:10,000) (Fisher), then immediately rinsed and washed 3 times for 5 minutes with 0.2% PBS Triton followed by a post fix of 4% PFA. Antibodies used were rabbit anti-dpERK (Cell Signaling) at 1:100. Secondary antibodies used were: Alexa Fluor-488nm-conjugated goat anti-mouse, Alexa Fluor-568nm goat

anti rabbit, Alexa Fluor-488nm donkey anti-sheep (all 1:1250), and DAPI (1:10,000).

Microscopy:

Egg chambers were imaged with a Leica DM2500 compound light microscope at 100, 200, or 400x and QCapture image software. Confocal images were acquired using the Leica sp8. All z-stacks were max projected. Eggs for imaging via scanning electron microscopy were collected on agar plates and mounted on aluminum SEM stubs with double-sided carbon tape. The stubs and eggshells were then immediately sputter coated with gold/palladium with a Denton Vacuum Desk II sputter coater before imaged using LEO 1450EP at high vacuum ($<1x10^{-5}$ torr). Captured images were rotated, cropped, and prepared using NIH ImageJ.

Quantification of GRK gradient

The GRK gradient was measured as the average intensity of GRK staining in dorsal views of stage 10A egg chambers (Fig. 8). Images were imported into ImageJ and pixel intensities were measured by placing a line (thickness =75 pixels) positioned after the nucleus (Fig. 8B). The average of these plot profiles were normalized such that the highest intensity GRK staining were equal to 1.00, with all subsequent values represented as a fraction relative their intensity and plotted with standard error bars. Using the Leica SP8 confocal microscope, maximum intensity was performed on z stacks of all images used.

Quantification of changes in Broad patterning

Broad (BR) patterns were quantified by comparing the average number of non-BR expressing cells/ BR expressing cells in dorsal views of stage 10a egg chambers (Fig. 12A). The width of the box, was 10 cells counted going dorsal to ventral, a box was then positioned to end at the collar of the dorsal clearing, and at the most posterior of the dorsal clearing. A cartoon (Fig.12A) shows the wild-type dorsal clearing of BR, and the corresponding calculation (Fig.12B). The ratios were plotted with standard error bars. A two-tailed T-test was performed on these ratios ($p<.05$)

Results

Perturbations of *dally* shape GRK gradient

In wild type flies, GRK, at stage 10A, is secreted from the oocyte at the dorsal anterior and is seen as a gradient along the dorsal midline extending about 50% of the oocyte (Fig. 9A). Dally has been shown to play major roles in shaping morphogen gradients, so we proposed that over expressing of Dally would restrict the GRK signal (Lin, 2004). When *dally* was overexpressed the GRK gradient showed narrower gradient, corresponding an extension by approximately two cells (Fig. 10). When you deplete *dally,* GRK should become

less restricted, GRK Showed a more diffuse gradient which resulted in an approximately 2 cells decrease (Fig. 10).

EGFR signaling activation in perturbations of *dally*

We next tried to see if the shaping of the GRK gradient corresponded to changes in activation of the EGFR signaling pathway, through dpERK staining. Grk binds to the EGFR receptor *torpedo,* activating the canonical Ras-Raf-MEK pathway. In overexpression of *dally,* the GRK gradient was narrower, which should correspond to a more restricted dpERK staining, which is what see (Fig. 11B). In under expression of *dally* the dpERK corresponds to a more diffuse GRK gradient, as seen by the less defined dpERK staining.

Tissue patterning is regulated by Dally

At stage 10A egg chambers, high levels of EGFR controls the clearing of Br in the dorsal midline (Deng and Bownes 1997). If Dally is regulating the shape of the GRK gradient, and subsequently, reducing activation of EGFR signaling, then we should see a reduced clearing of BR in the dorsal midline. In overexpression of *dally* a reduced clearing of BR in the dorsal midline was, where we reported a ratio of non-BR to BR expressing cells at (.43), as compared to wild type (.59) (Figs. 13A, 14), while in depletion of *dally* only a minor narrowing of the clearing of BR, (.52). (Figs. 13B, 14).

Effects of mutant Dally on EGFR signaling

Dally is linked to the membrane via a glycosylphosphatidyl inositol (GPI) linkage. Using a UAS- ΔGPI line we ectopically expressed a Dally protein lacking the GPI anchor, which causes for a “free-floating” form of Dally. Interestingly, the GRK pattern did not extend beyond the nucleus. (Fig. 10A). Consistent with the truncated GRK gradient, this form of Dally showed defects in the patterning of BR. Specifically, in the dorsal midline, there were BR expressing cells, which were not repressed as seen in wild-type BR patterning.

Eggshell morphology is disrupted by *dally* perturbations

Next, we were interested to determine how change in Dally affect eggshell morphologies. It has been shown that eggshells with reduced EGFR signaling are ventralized, with single appendage phenotypes (Schüpbach, 1987). Overexpression of *dally* generated 30% of eggshells with no gap between the DAs (phenotype I), where as in wild type it is only 8% (Fig. 16). In expression of the “free-floating”, ΔGPI form of Dally, we observed 18% of eggshells resulted phenotype I (Fig. 16B). Also, in the mutant form of Dally generated a more severe phenotype categorized as phenotype II (Fig. 16C). In depletion of *d*ally, there were no significant changes observed.

Discussion

HSPGs in development

HSPGs have been shown to interact with several secreted signaling molecules that are involved in metazoan development, including members of the

Wnt/Wingless (WG), Hedgehog (Hh), transforming growth factor-β (TGFβ) and fibroblast growth factor (FGF) families (Han *et a*l., 2004; Lin, 2004). Glypicans represent the main cell-surface HSPGs that are linked to the plasma membrane by GPI linker. In *Drosophila*, *dally,* has been shown to be involved in cell division, development of the visual system, morphogenesis of the eye, wing, and genitalia (Nakato *et al*., 1995). An intriguing finding about *Dally* is that it has different functions in different tissue. For example, *dally* mutant phenotypes are enhanced by decreasing Dpp in the eye, antenna, and genitallia, but the opposite is in the wing where dpp mutations are suppressed (Jackson *et al.* 1997). Here, we show that Dally interacts with EGFR signaling as well.

During oogenesis, the EGFR pathway defines the DV axis, which then patterns the follicle cells into a 3D structure. GRK levels in the dorsal midline determine the patterning clearing of BR, for EGFR is required for BR activation in the roof domain and BR repression in the dorsal midline (Yakoby *et al*., 2008). If GRK, and subsequently, downstream levels of EGFR are being affected by Dally, then tissue patterning of the BR should be affected. In concordance with this prediction, the narrowing of the GRK gradient in over expression of dally, corresponded to a decrease in the dorsal clearing of BR (Figs.13D,14). These patterning effects can be understood by when *dally* is overexpressed, the concentration of GRK is higher near the source, however declines more rapidly (green curve) as compared to wild type GRK gradient (red curve) (Fig. 18). This more rapid decline results in lower levels of GRK away from the source resulting in reduced EGFR activation and patterning (green cells) (Fig 18). In depletion of

dally, the GRK concentration is lower (blue curve) than in wild type, which, also leads to reduced EGFR activation and patterning (blue cells) (Fig. 18). Based upon our analysis of the GRK gradient, Dally seems to be having a stabilizing effect on the diffusion of GRK, which corresponds to Dally's interaction with other signaling pathways (Lin, 2004; Yan and Lin, 2009). .

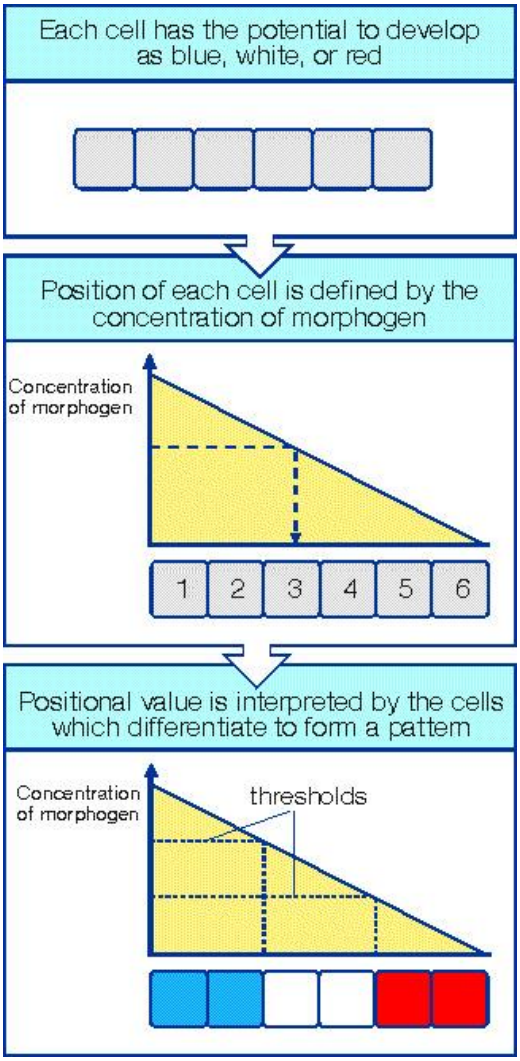
Dally is required for EGFR signaling

A number of studies have also shown that the HSPGs core protein are important in morphogen distribution, for they can modify HS chains, as well as directly involved in cell signaling (Esko and Zhang, 1996; Capurro *et al*., 2008). When expressing a uniform Dally protein mutant for the GPI anchor, we saw a reduced GRK gradient (Fig.15A). Remarkably, in the ΔGPI mutants the GRK staining could only be seen around the nucleus, and not in the extended trail seen in wild type (Fig. 7B). The lack of the GPI anchor seems to stunt the GRK gradient, suggesting that this "free-floating" form of Dally is sequestering GRK away from its receptors. These results are consistent with BR patterning in these perturbations (Fig.15B), where BR was seen in the dorsal midline.

Eggshell patterning has been studied extensively in *D*. *melanogaster.* (Berg, 2005, Deng and Bownes, 1997; Ruohola-Baker *et al.,* 1993; Tzolovsky *et al.,* 1999). The dorsal clearing of BR is controlled by EGFR signaling, and corresponds to the gap seen between the appendages (Pizette *et al*., 2009) In overexpression of *dally* reduced EGFR signaling phenotypes were observed (Fig.16B). In depletion of *dally,* no noticeable phenotype was found, however in the GPI mutants we saw reduced EGFR phenotypes, including severely

ventralized eggshells (Fig.16C). When Dally is not anchored to the plasma membrane, GRK levels are below threshold of EGFR activation resulting in reduced EGFR phenotypes (Fig. 16C). Although, in depletion of *dally,* we see a more diffuse GRK gradient and dpERK patterns, the levels of activation are still optimal to split the DAs. Studies of Hh, Wg, and dpp morphogens in the wing disc have shown that restricted diffusion is HS-mediated (Yan and Lin, 2009). Preliminary results for the ΔHS mutants have eggshell phenotypes similar to the mutant GPI flies, results not shown. According to these results, Dally is required for the formation of the GRK gradient, and thus optimal EGFR activation.

The interaction between the BMP and EGFR pathways during eggshell patterning is a complex and must be fine-tuned. Small changes in the gradients of both Dpp and GRK can change the eventual morphology of the eggshell (Peri and Roth, 2000; Ruohola-Baker *et al.,* 1993; Schüpbach, 1987; Tombly *et al.,* 1996). Dally has been shown to be induced, as well as regulated by BMP during oogenesis (Lemon and Yakoby, unpublished. Here, we propose a model, where Dally is the coordinator between the EGFR and BMP signaling pathways during oogenesis (Fig. 19). Interestingly, EGFR signaling has been shown to negatively regulate *dally* in the ovary, therefore it is possible that, in the follicle cells, *dally* may also be regulated by EGFR signaling. (Matsuoka *et al*., 2013).



Wolpert and Tickle, Principles of Development, 4th Edition. 2001.

Figure 1. Morphogen Gradients

Cells have the ability to differentiate into either red, white or, blue cells. What determines a cells fate is the concentration gradient of the morphogen. Using this theory, a group of identical cells can form patterns as seen above as the “French flag model”. (Turing, 1952; Wolpert, 1969).

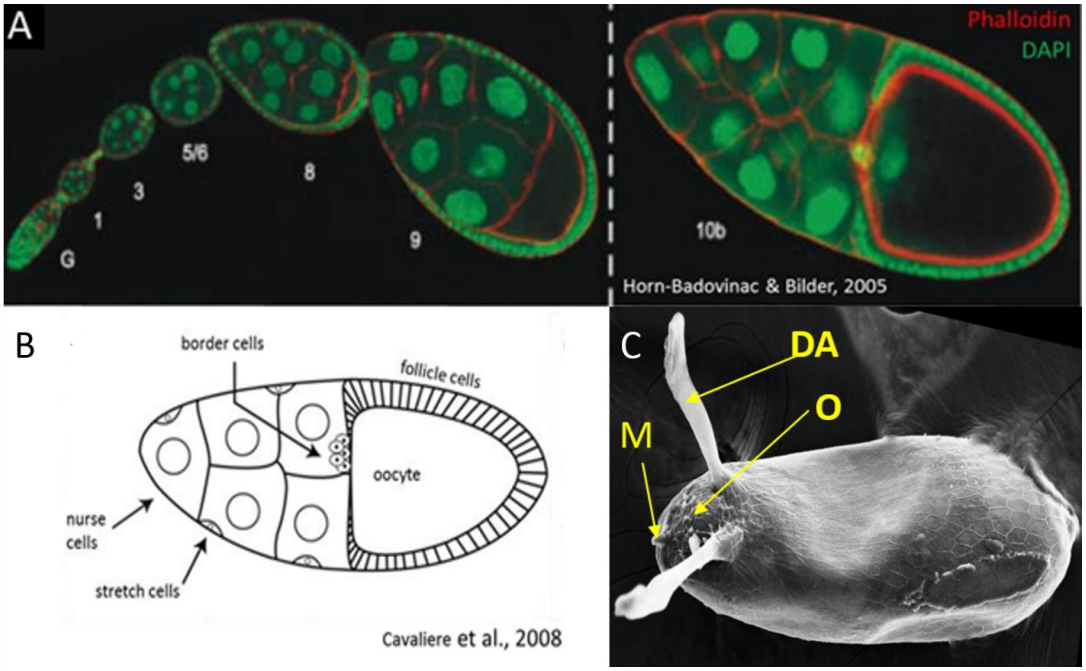
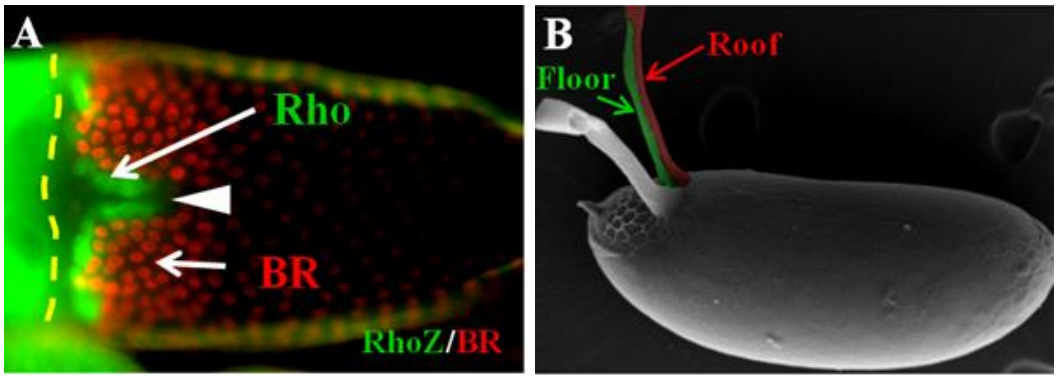


Figure 2. *Drosophila melanogaster oogenesis*

A) Egg chambers stages 1 through 10B of *D. melanogaster* oogenesis (nuclei are labeled DAPI, green, membranes labeled with phalloidin, red)

B) Cartoon stage 10b egg chamber. Oocyte is surrounded by follicle cells (FC). Nurse cells to the anterior supply the oocyte with nutrients, are surrounded by epithelial cells called stretch cells.

C) Electron microscopy of *D. melanogaster* eggshell. Two tubular structures called Dorsal Appendages (DA), which allow for gas exchange, operculum (O), allows for larva to escape, and Micropyle (M), which, allows for fertilization.



(Niepielko et al., 2012)

Figure 3. Tissue patterning during oogenesis

A) Broad (BR) expressing cells (red), form the roof of the DAs (red arrow) (B), and the rhomboid (Rho) expressing cells (green) form the floor of the DAs (green arrow) (B). Dorsal midline (arrow head).

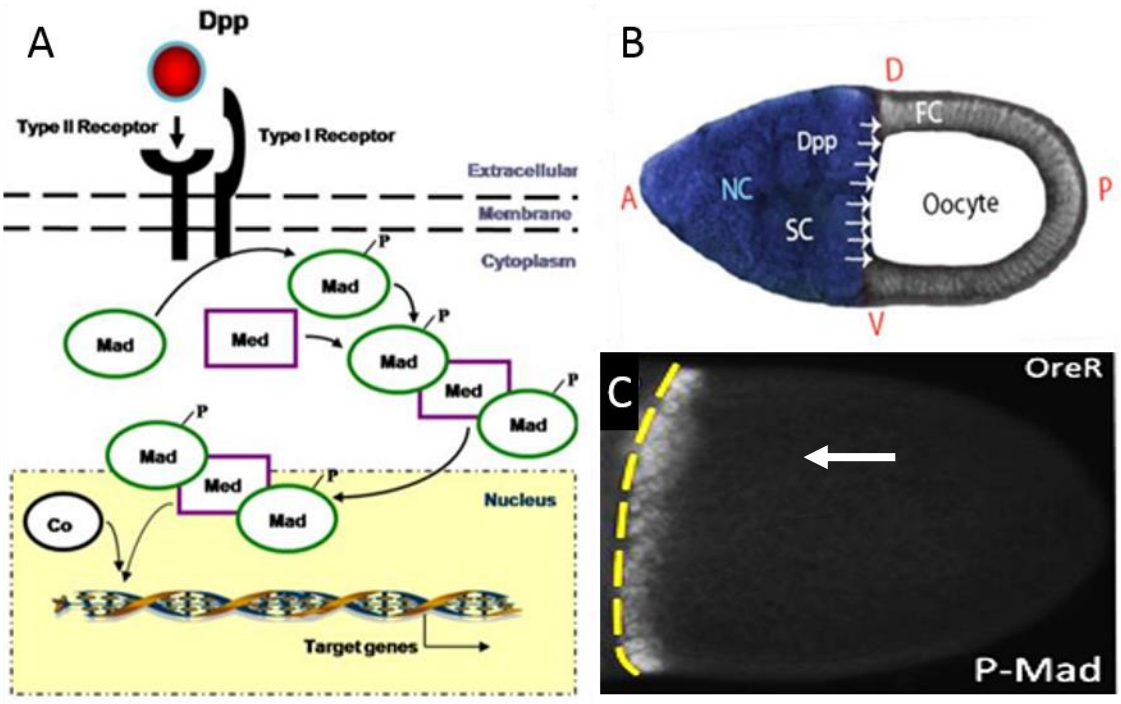
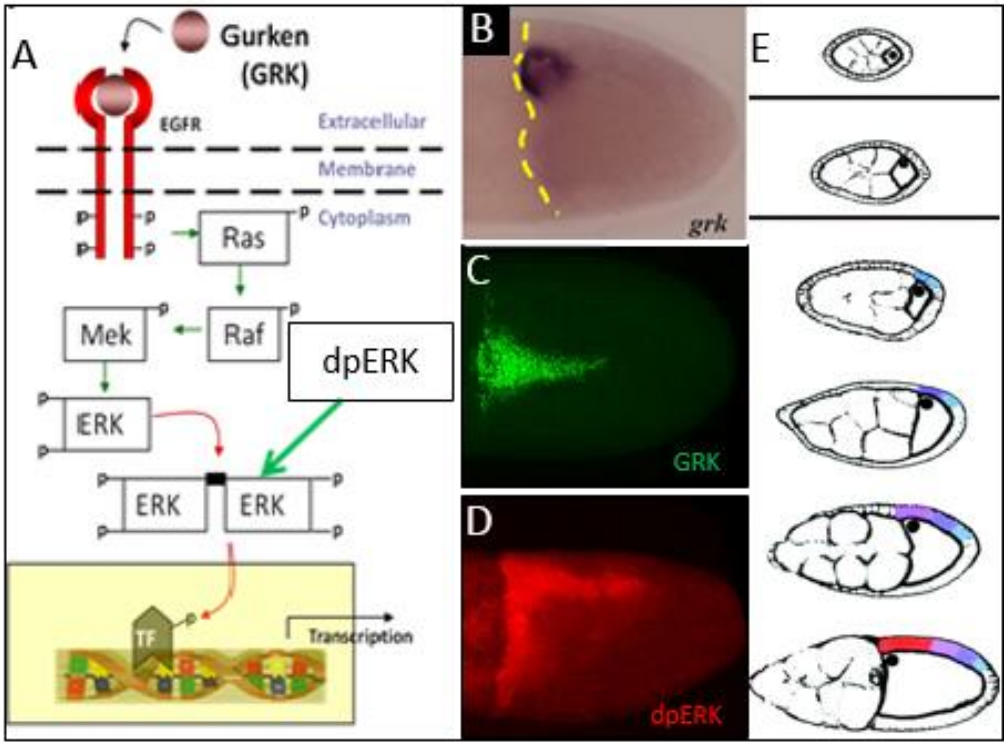


Figure 4. Bone morphogenic protein (BMP) signaling during oogenesis

A) Decapentaplegic (Dpp) binds to heteromeric receptor complex which then the type I receptor phosphorylates SMAD to make P-MAD, two P-Mad bind to one Medea (MED), and with other proteins enter the nucleus to control gene expression.
B) Dpp is secreted from the stretch cells and anterior most centripetally migrating follicle cells to generate a gradient from anterior to posterior.
C) Wild-type, Stage 10A, egg chamber shows P-Mad staining 2-3 rows of cells from anterior (yellow line). (Dorsal marked with green arrow).



(Van Buskirk and Schüpbach, 1999)

Figure 5. Epidermal growth factor receptor (EGFR) signaling during oogenesis

A) In *Drosophila* oogenesis the TGF-alpha- like ligand Gurken, binds to the EGF receptor activating the canonical Ras-Raf-Mek pathway.
B) *grk* RNA tightly associated with oocyte nucleus (lateral view)
C) GRK at stage 10A (green) (dorsal view)
D) EGFR signaling monitored through dpERK, stage 10A (red) (dorsal view)
E) During early stages of oogenesis (prior to stage 9) the oocyte nucleus is positioned posterior end, there GRK is secreted and defines the AP axis. During later stages, the oocyte nucleus travels to the dorsal-anterior of the oocyte where GRK signaling sets the DV fates.

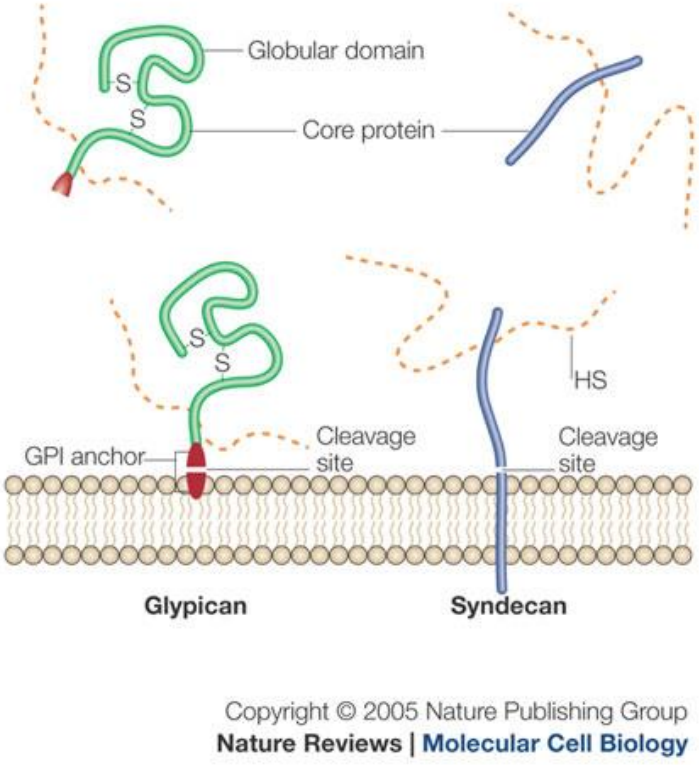
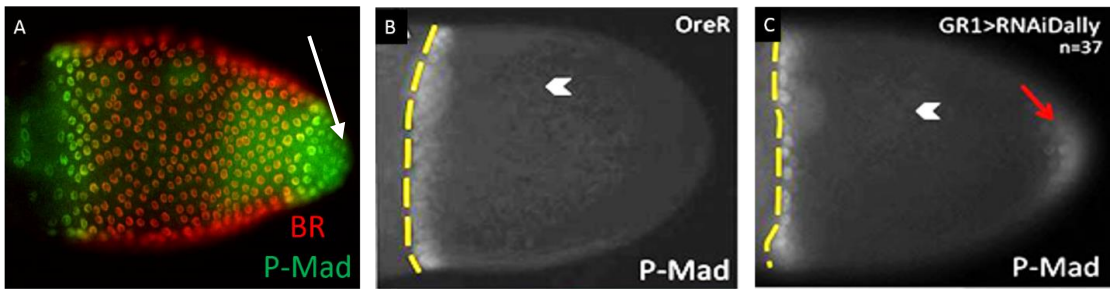


Figure 6. Heparan sulfate proteoglycans (HSPGs)

HSPGs are extracellular molecules (ECM). They are comprised of a core protein to which heparan sulfate (HS) glycosaminoglycan (GAG) chain are attached, and are anchored to the plasma by glycosylphosphatidyl inosoitol (GPI) linkage. Two glypicans have been identified in *D. melanogaster* division abnormally delayed (Dally), and Dally-like protein (Dlp).



(Yakoby, unpublished)

(Lemon and Yakoby, unpublished)

Figure 7. Evidence that Dally is affecting EGFR pathway in ooge

A) In GRK null background flies, ectopic P-Mad is seen in posterior of egg chamber (white arrow).
B) In wild-type egg chambers (stage 10A), P-MAD is seen in 2-3 rows from the anterior domain (yellow line). Dorsal marked by white chevron.
C) In depletion of *dally* ectopic P-MAD is seen in posterior (red arrow) suggesting a reduction in EGFR signaling.

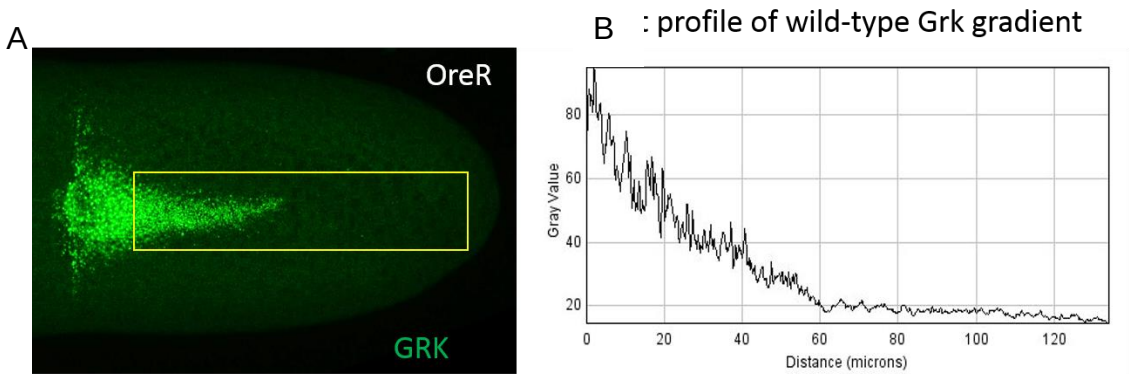


Figure 8. Quantification of GRK gradient

A) Dorsal view of stage 10A egg chamber, stained for GRK. Average staining intensities of yellow box were plotted over distance toward posterior. Boxes were placed over dorsal anterior as stated in Quantification of GRK Intensities, Materials & Methods.
B) Plot profile from sample yellow box (A), calculated in Imagej. Average GRK intensities from all egg chambers plotted in Fig 8.

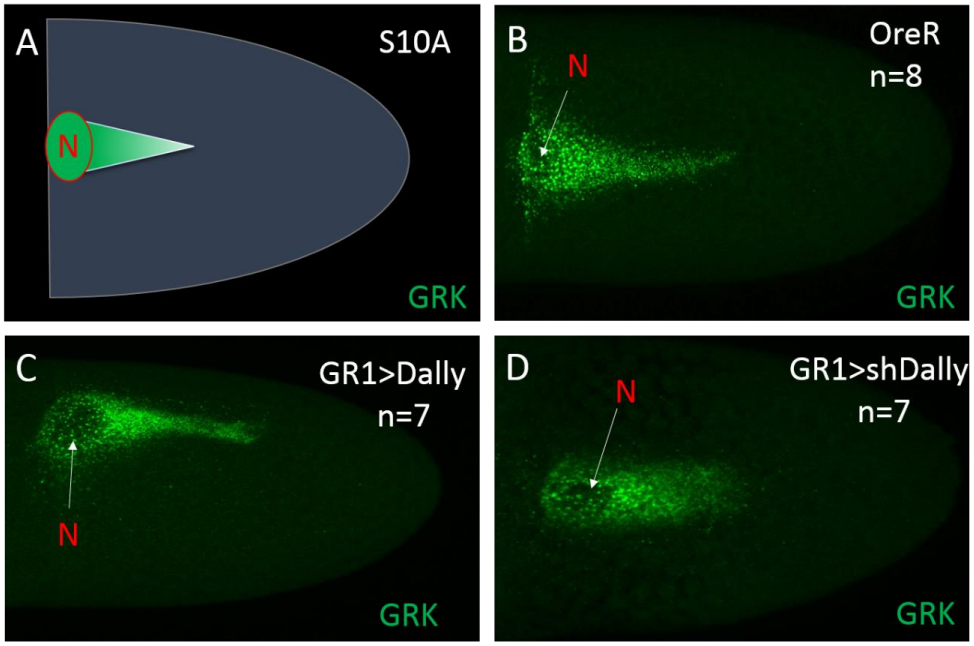


Figure 9. Dally perturbations affect GRK gradient

A) Schematic showing dorsal view of GRK gradient at stage 10A

B) Wild-type GRK gradient was quantified in graph 1. At stage 10a, GRK reaches approximately 12 cells of the oocyte, or 50%. (Line marks end of nucleus).

C) Uniform overexpression of *dally* resulted in a longer, narrower GRK gradient.

D) Uniform depletion of *dally* showed a shorter, diffuse GRK gradient.

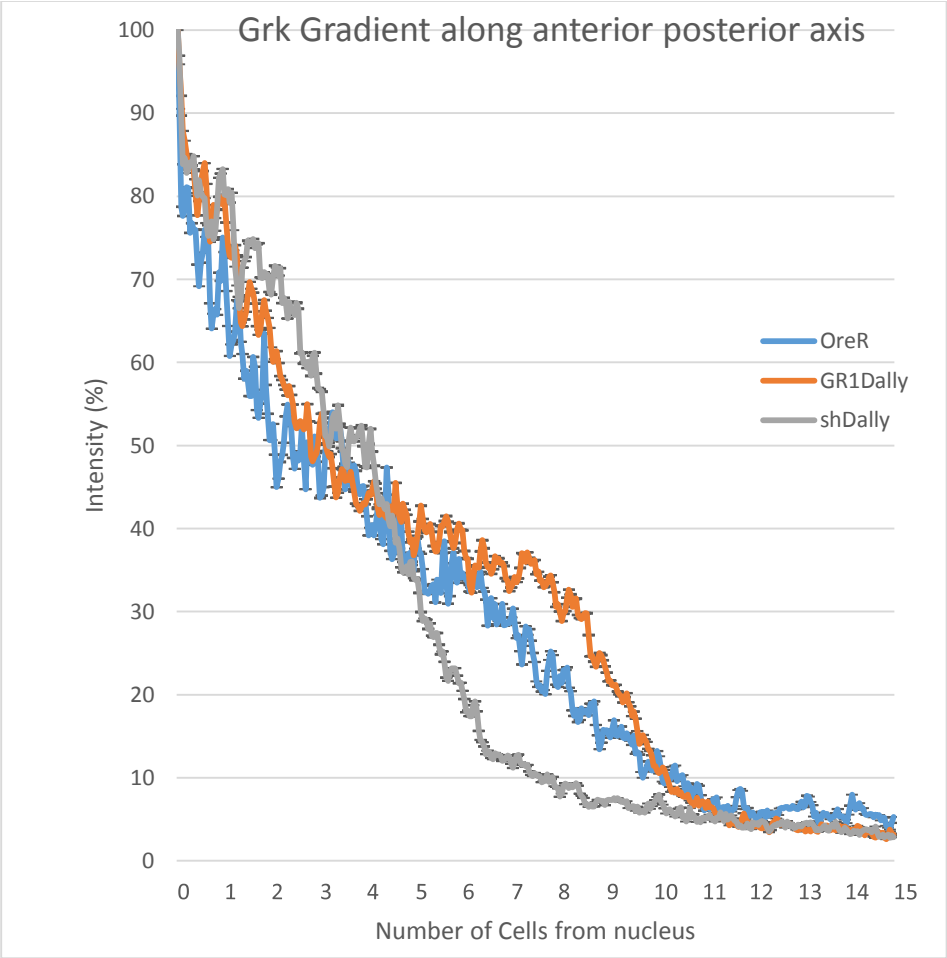


Figure 10. GRK intensities in *dally* perturbations

Relative intensity GRK staining of ectopic overexpression and under expression of *dally.* Over expression of *dally* shows an increase in GRK expression of approximately two cells (Blue line). Depletion of *dally* results in a decrease in GRK by approximately two cells (Grey line).

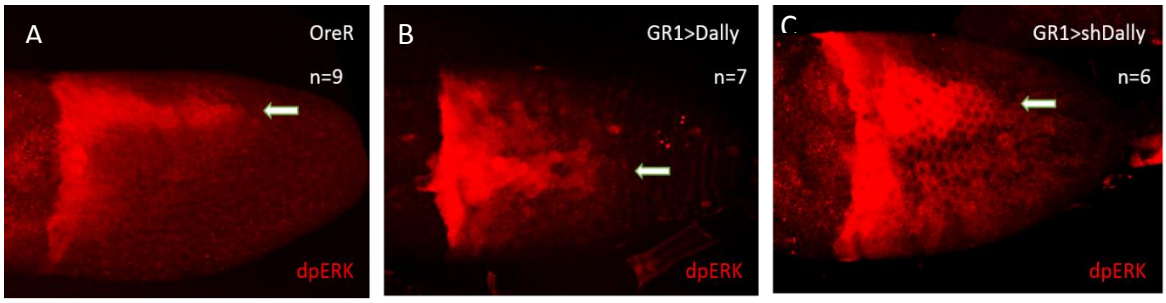


Figure 11. Dally shapes dpERK patterns

A) Wild type dpERK during stage 10A, (Dorsal views, marked by white arrow)
B) Over expressing Dally throughout the follicle cells the follicle cells changes these dpERK pattern to be more restricted.
C) Depletion of *dally* shows a more diffuse dpERK signaling.

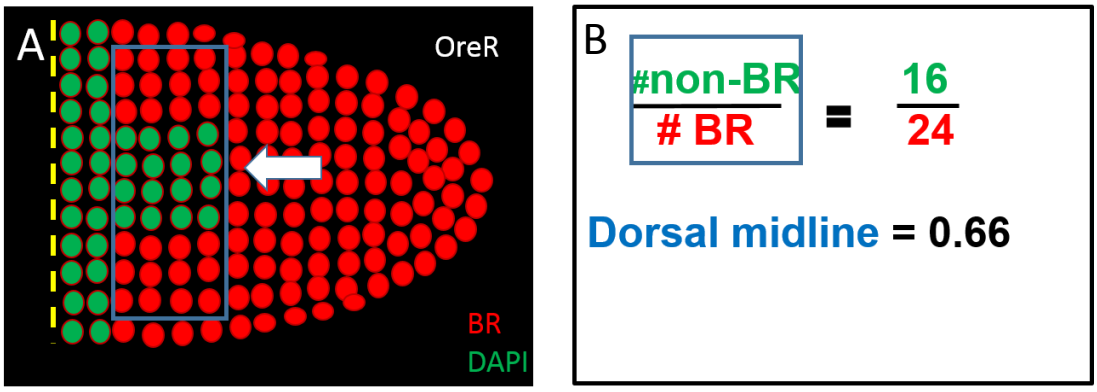


Figure 12. Quantification of dorsal midline

A) Schematic of BR (red) expressing egg chamber showing midline clearing (green). Midline was counted as the ratio of non-BR expressing cells to BR expressing cells counted in blue box (created from specification in Materials and methods). White arrow marks dorsal midline. Anterior domain marked by yellow arrow.
B) Example of Wild-type midline calculation. An increase in ratio would suggest a widening of the dorsal midline, where as a decrease in ratio would suggest a decrease in dorsal midline.

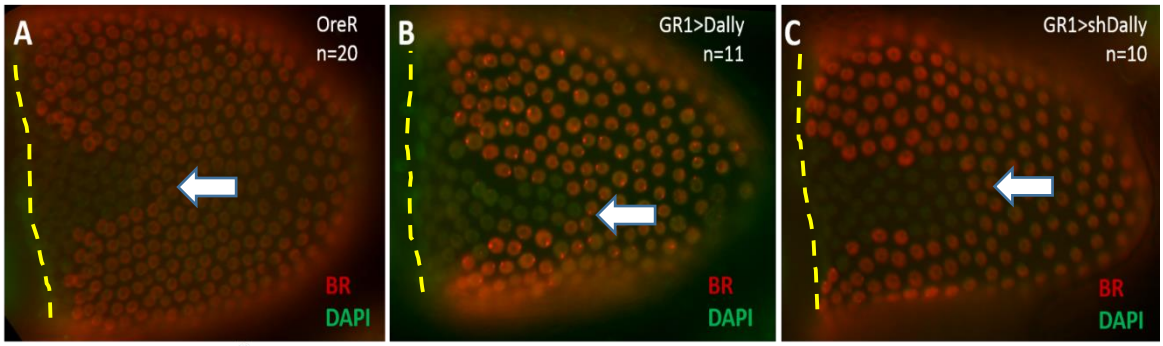


Figure 13. Tissue patterning effects seen in *dally* perturbations

A) Wild-type BR shows a midline gap. Wild-type BR expression shows a width of approximately 4 cells. White arrow marks dorsal midline. Anterior domain marked by yellow line.

C) Over expression of *dally* leads to a narrowing of the dorsal midline.

D) Depletion of *dally* resulted to slight narrowing of the dorsal midline.

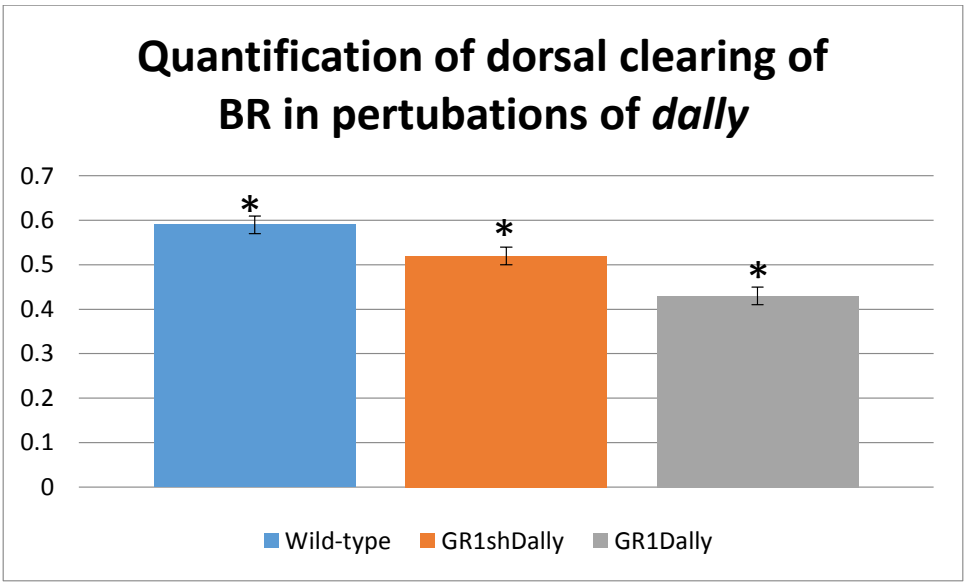


Fig 14. Quantification of dorsal clearing of BR in perturbations of *dally*

Dally perturbations lead to changes in dorsal clearing of Br. Ratio of non BR expressing cells to BR expressing cells. Overexpression of *dally* shows a decrease in width of dorsal clearing of Br cells and depletion of *dally* shows a small decrease in width of dorsal clearing. Error bars express standard errors, asterisks represent significant differences in raw data averages when compared to OreR ($p<.05$).

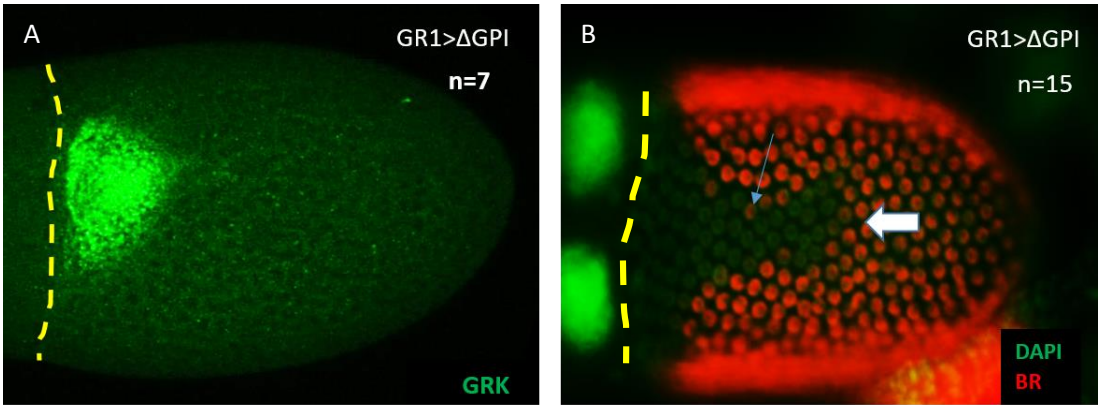
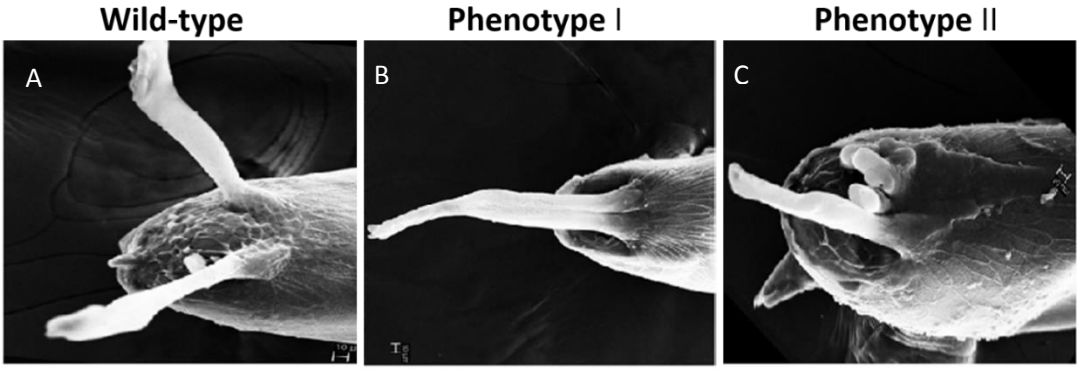


Figure 15. Mutant Dally protein results in reduced GRK distribution

A) In ΔGPI mutants, GRK staining did not have an extension beyond the nucleus, which is normally seen in wild-type GRK. White arrow marks dorsal midline. Red arrow marks nucleus. Anterior domain marked by yellow line.

B) Lack of GRK signaling causes an irregular dorsal clearing of BR, as shown by BR cells in the dorsal midline (Blue arrow). White arrow marks dorsal midline. Anterior domain marked by yellow line.



D	Wild-type	Phenotype I	Phenotype II	n (total # counted)
OreR	243	23	0	266
%	91%	9%	0	
GR1>Dally	126	45	0	178
%	70%	30%	0	
GR1>ΔGPI	150	35	8	193
%	78%	18%	4%	
GR1>shDally	130	8	0	138
%	94	6%	0	

Figure 16. Eggshell phenotypes by perturbing *dally*

A) Wild type eggshells

B) An increase in eggshells with phenotype I were seen in overexpression of *dally* (30%), and in ΔGPI (18%), as compared to wild type (9%). Under expression of *dally* resulted in (6%).

C) In expression of the ΔGPI of Dally we observed eggshells with phenotype II (3%).

D) Table with counts of phenotypes (Wild type, Phenotype I, II).

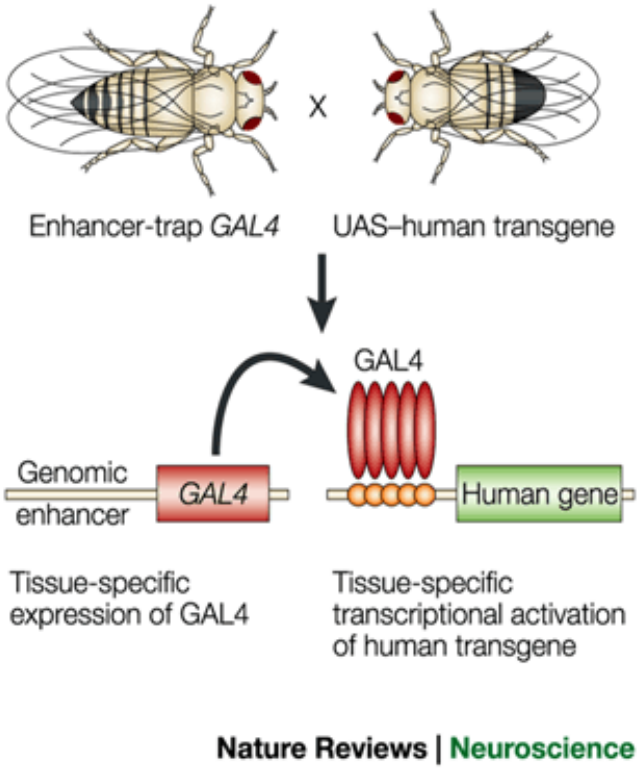


Figure 17. GAL4/UAS system for targeted gene expression

The GAL4/UAS system allows for targeted gene expression in *Drosophila*. Diagram showing the fly containing the Tissue specific enhancer-trap GAL4, a yeast transcription factor is crossed to fly with an upstream activating sequence (UAS) to a gene or construct of interest. The progeny will express the gene or construct of interest in a tissue specific manner according to the GAL4 used.

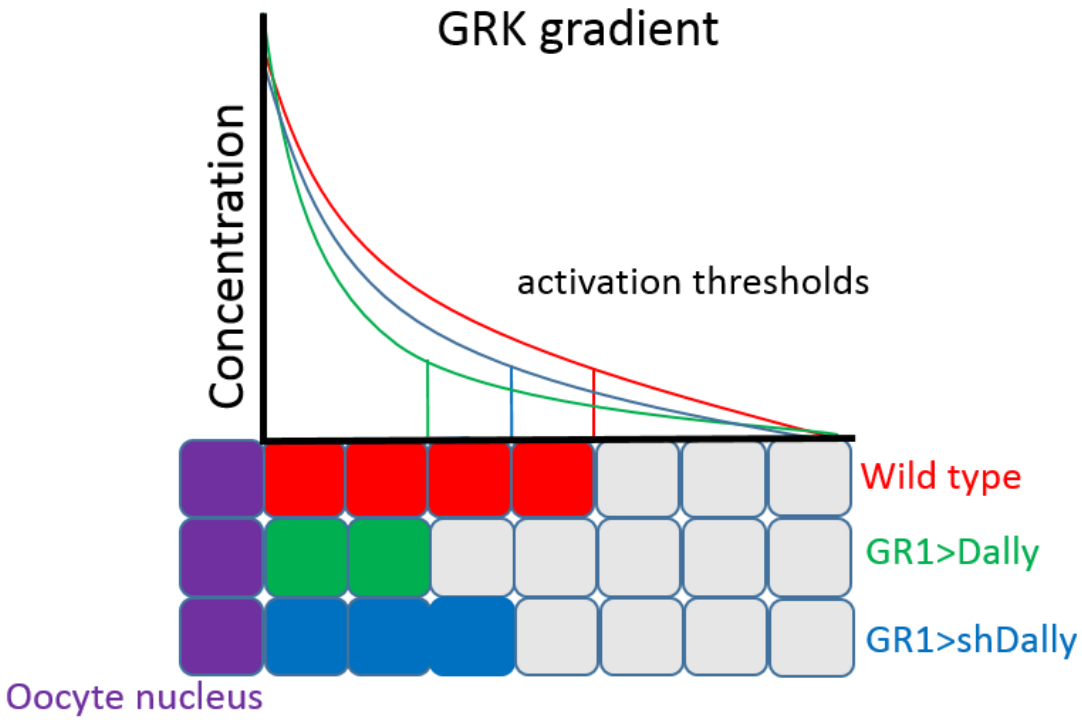


Figure 18. Dally's effect on GRK gradient formation, and activation of EGFR signaling

A cartoon showing how Dally is required for the formation of the GRK gradient which then activates optimal levels of EGFR signaling. In wild type, the GRK gradient reaches activation thresholds patterning 4 cells (red). In overexpression of *dally* the GRK gradient is restricted near the source, leading to a more rapid decline of GRK levels (green line), this leads to the patterning of 2 cells (green boxes). When *dally* is depleted, the GRK gradient becomes more diffuse (blue line) resulting in the patterning of 3 cells (blue boxes).

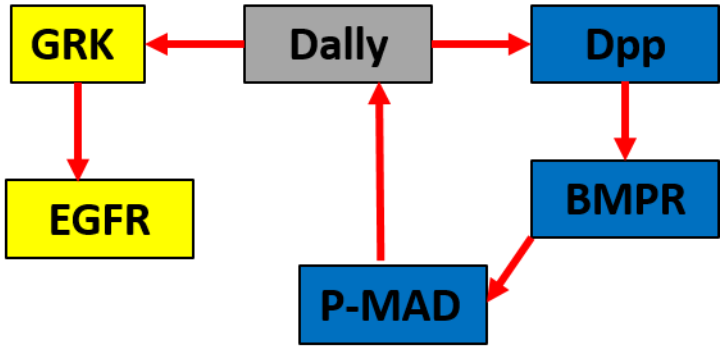


Figure 19. Coordination of EGFR and BMP signaling through Dally

Along with Dally regulating the BMP signaling pathway, here include Dally as a coordinator between both EGFR and BMP during *D. melanogaster* oogenesis. Dally is involved in shaping the gradient of both GRK and DPP in the follicle cells (Lemon and Yakoby, unpublished).

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