Systems-level questions in *Drosophila* oogenesis

N. Yakoby, C.A. Bristow, I. Gouzman, M.P. Rossi, Y. Gogotsi, T. Schüpbach and S.Y. Shvartsman

Abstract: This paper describes computational and experimental work on pattern formation in *Drosophila* egg development (oogenesis), an established experimental model for studying cell fate diversification in developing tissues. Epidermal growth factor receptor (EGFR) is a key regulator of pattern formation and morphogenesis in *Drosophila* oogenesis. EGFR signalling in oogenesis can be genetically manipulated and monitored at many levels, leading to large sets of heterogeneous data that enable the formulation of increasingly quantitative models of pattern formation in these systems.

1 Introduction

Cell communication mediated by diffusible signals is essential for pattern formation in embryonic development. In one of the most common pattern formation mechanisms, a group of cells secretes a ligand that spreads through the tissue, binding to and activating cell surface receptors. The resulting gradient of receptor activation governs gene expression across the patterned tissue. Such position-dependent control of gene expression by secreted molecules is called morphogenetic signalling [1]. One striking conclusion of genetic studies in multiple organisms is that the same morphogen ligands, receptors and signalling pathways are used both across different organs within the same organism and across species. We study the epidermal growth factor receptor (EGFR), a key regulator of developing tissues in animals from worms to humans.

EGFR belongs to a large class of receptor tyrosine kinases [2, 3]. EGFR is activated when a ligand binds to its extracellular domain, inducing receptor dimerisation and subsequent phosphorylation of the receptor's cytoplasmic tail [4]. The phosphorylated receptor activates intracellular signalling that controls gene expression and cell behaviours such as differentiation or migration. Abnormal EGFR signalling is associated with severe developmental defects and many types of cancer [2, 3]. Many molecules mediating the EGFR-induced responses became drug targets in oncology and other areas of medicine. However, neither the contribution of EGFR to tissue morphogenesis in development nor the exact roles of deregulated EGFR signalling in diseases are understood at this time. The key challenge in the analysis of the EGFR function *in vivo* is to integrate the existing molecular and cellular information into a systems-level description of the EGFR network in tissues.

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We are using the fruit fly *Drosophila melanogaster* as an experimental system for the development and validation of quantitative models of EGFR signalling in tissues. In addition to the sequenced genome and a wealth of cellular and molecular data available in *Drosophila*, there are specific reasons that make the fruit fly an attractive system for the study of EGFR signalling *in vivo* [5, 6]. The fruit fly EGF receptor is homologous to its human counterpart. It is activated by four different ligands, all of which have mammalian homologues, implicated in multiple diseases. The intracellular pathways mediating EGFR signalling are also conserved between humans and fruit flies. Alterations in *Drosophila* EGFR signalling produce distinct morphological phenotypes at all developmental stages, from the egg to the adult. Finally, versatile genetic tools enable highly specific manipulation of the *Drosophila* EGFR network. Here, we describe the modelling, experimental and data integration issues associated with systems-level analysis of EGFR signalling in the development of the *Drosophila* egg.

2 EGFR signalling in *Drosophila* egg development

The mature fruit fly egg is a highly asymmetrical, three-dimensional structure [7]; see Fig. 1*a*. Its most prominent features are the point for sperm entry (the micropyle), the region from which the larva hatches (the operculum) and a pair of respiratory appendages supplying the embryo with oxygen. Remarkably, the polarity of the eggshell is transmitted throughout all stages of development (e.g. the dorsal side of the eggshell corresponds to the dorsal side of the embryo and the adult fly).

The elaborate structure of the eggshell is a result of cell–cell communication and pattern formation within the epithelial layer that envelops the egg chamber [8]. The egg chamber, which develops in the fly ovary, is composed of the oocyte, 15 nurse cells that supply the oocyte with mRNA and proteins, and an epithelial layer of ~1000 cells, called the follicular epithelium [7], Fig. 1*b*. In oogenesis, this layer of initially identical cells is patterned, i.e. cells within the layer express genes in a spatially non-uniform way. EGFR signalling plays a key role in patterning of the follicular epithelium, establishing the asymmetry of the eggshell and inducing the axes of the embryo [9]. Specifically, the EGFR ligand Gurken is released from the oocyte and induces multiple cell fates in the overlying

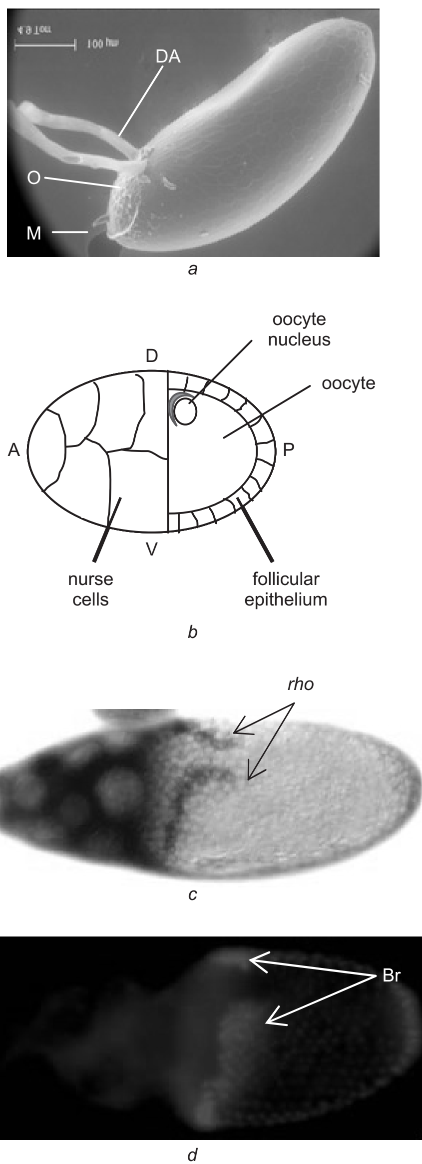

Fig. 1 *ESEM image of mature Drosophila eggshell*

a Dorsal appendages (DA), operculum (O), micropyle (M)
b Schematic diagram of different cell types in egg chamber, precursor of mature egg
Anterior (A), posterior (P), dorsal (D), ventral (V)
c Rhomboid (*rho*), known transcriptional target of EGFR in follicular epithelium, is expressed in two 'L-shaped' spatial patterns
d Expression pattern of Broad protein, known target of EGFR signalling, appears as two dorsolateral patches

follicular epithelium. Locally secreted Gurken activates EGF receptors that are uniformly distributed across the follicular epithelium and acts as a morphogen in the patterning of this epithelial layer [10, 11].

3 Pattern formation in the follicular epithelium

Gurken patterns the eggshell and specifies both the anteroposterior and the dorsoventral axes of the embryo by regulating gene expression in the follicular epithelium, Figs. 1*c* and *d*. For example, *rhomboid1* (*rho*) and Broad (*br*) are two of the genes induced by Gurken/EGFR signalling. *rho* and *br* are expressed in adjacent and non-overlapping groups of dorsal follicle cells that later contribute to different parts of future dorsal appendages [12–15]. Pipe (*pip*) is an example of a gene repressed by Gurken; it is expressed in the ventral follicle cells and is essential for the ventral patterning of the future embryo (reviewed in [16]).

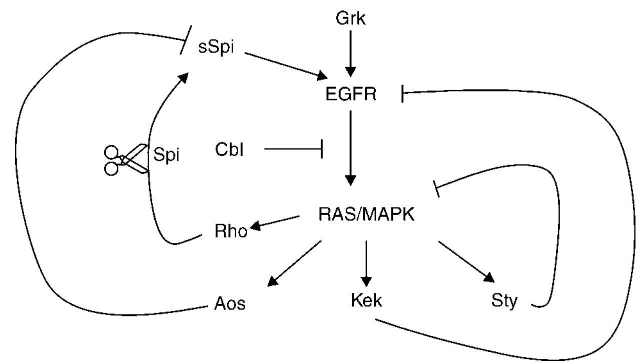

Fig. 2 *Diagram of feedback loops that modulate EGFR signalling in follicle cells*

Analysis of morphogenetic signalling in multiple experimental systems has revealed that the initial response of a cell to a morphogen can be followed by the activation of feedback loops that generate increasingly complex patterns of gene expression in the field of responding cells [1, 17]. The Gurken morphogen induces two positive and three negative feedback loops that modulate EGFR activity in the follicular epithelium, see Fig. 2. Both of the positive feedbacks involve EGFR-mediated release of EGFR ligands. Specifically, EGFR activation by Gurken induces the expression of *rho*, an intracellular protease crucial for intracellular processing, and secretion of Spitz, the most potent ligand of *Drosophila* EGFR [18, 19]. The second positive feedback depends on transcriptional activation of Vein, another ligand of EGFR, believed to be weaker than Gurken and Spitz [19]. The first of the negative feedback loops relies on Argos, a secreted protein that sequesters EGFR ligand Spitz [20, 21]. The second negative feedback loop depends on Kekkon1, a transmembrane protein that inhibits ligand-induced EGFR dimerisation [22, 23]. The third negative feedback loop is mediated by Sprouty, a cytoplasmic protein that inhibits signal transduction downstream of activated EGFR [24]. All three inhibitors are induced by EGFR and expressed in a similar spatiotemporal pattern. Understanding the roles of these overlapping feedback loops in follicle cell patterning is one of the main goals in the development of the systems-level model of oogenesis [25, 26].

4 Morphogen gradient and feedback mechanisms

Genetic analyses of *Drosophila* oogenesis have led to two conceptual models that aim to explain how the Gurken gradient is transformed into a complex pattern of cell fates in the follicular epithelium. In the morphogen gradient model, pattern formation relies on a cell-autonomous mechanism, whereby gene expression in the follicle cells is determined by the dose of Gurken-mediated activation of EGFR [27]. In the feedback model, follicle cell patterning depends on the non-cell autonomous positive and negative feedback loops (through Spitz and Argos) that spatiotemporally modulate the Gurken input [19].

These mechanisms were proposed on the basis of genetic manipulations that lead to quantitative and/or qualitative changes in the eggshell morphology and/or in the patterns of gene expression in the follicular epithelium. For example, increase/decrease in the dose of the oocyte-derived Gurken signal generates a wider/smaller gap between dorsal appendages and between the two stripes in the pattern of *rho* expression [27]. These effects are

consistent with the morphogen gradient model in which the 'interappendage' cell fate requires a high level of Gurken signal. The feedback model is supported by the experiments in which either positive or negative feedbacks have been eliminated, leading to eggshells with either one narrow or one broad appendage, respectively [19].

Both the morphogen gradient and the feedback models aim to describe the wild-type gene expression patterns as well as phenotypic transitions in the spatially distributed system with a large number of interacting components. Given the complexity of this system and the experimental difficulties associated with the monitoring of its dynamics, the evaluation of these mechanisms is not straightforward. For example, the feedback mechanism depends on the interaction between the positive feedback by Rhomboid/Spitz and the negative feedback by Argos. However, at present, the dynamics of these feedbacks cannot be monitored in real time. On the mechanistic level, one of the main systems-level questions in eggshell patterning is related to the role of cell–cell communication. Several experiments indicate that the coarse structure of the gene expression patterns can be explained by the cell-autonomous gradient interpretation mechanism, but the role of induced intercellular signals mediated by secreted molecules such as Argos, Spitz and Vein remains to be established.

At the most general level of description, it is important to investigate both the origin and the limits of robustness of the wild-type gene expression patterns and eggshell morphology. For example, 100% of eggs laid by wild-type females have the same morphology, and the underlying gene expression patterns are very robust. For example, the stripes in the *rho* expression are always just a single cell wide (Fig. 1*c*). It is necessary to investigate which mechanisms create this precision and how these patterns are destroyed in some of the mutants. Looking at eggshells of related fly species, it is natural to ask how the eggshell patterning system evolves. Related fly species have eggshells with more than two dorsal appendages [28–30] (see Fig. 3). At the same time, we know that these species also rely on Gurken and EGFR regulators in the follicle cells for patterning of the eggshell. Whether the knowledge derived from studies of oogenesis in *Drosophila melanogaster* can explain how more complex eggshell morphologies arise in other species must be ascertained.

5 Mathematical modelling of pattern formation

Mathematical modelling is an attractive approach to testing the consistency of the proposed mechanisms and making experimentally testable predictions [25]. In principle, the information about the regulatory interactions in *Drosophila* oogenesis can be used to formulate a mathematical model that can be analysed computationally to predict the dynamics of multiple components and the effects of genetic manipulations.

In the past, we have developed a mathematical model of the feedback (Rhomboid/Spitz/Argos) mechanism and, through extensive analytical and computational analysis of this model, demonstrated that it can account for a number of experimentally described phenotypic transitions, and predicted the emergence of more complex eggshell phenotypes [31–33]. Our main goal was to test whether the mechanism could account for the various eggshell morphology phenotypes. We were particularly interested in the phenotypes generated by the manipulations in the dose and the spatial distribution of the oocyte-derived signal. It is known that a systematic decrease in the level of Gurken signal can generate eggshells with one or zero dorsal appendages. At the same time, an increase in the dose leads to eggs with increased inter-appendage distance or one broad dorsal appendage. These observations provided constraints for our phenomenological model.

Analysis of this model revealed that the peak-splitting mechanism can be realised in one spatial dimension; see Fig. 4. This means that a single-peaked input in the

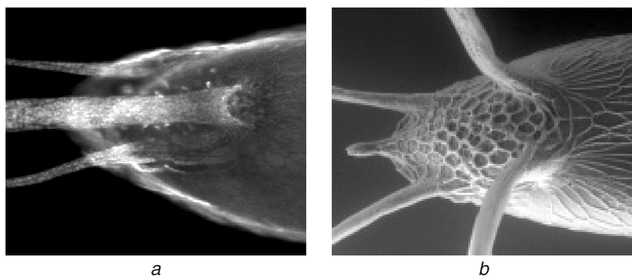

Fig. 3 *Eggshells of related fly species*

a Dark field image of *Drosophila phalerata* eggshell
b ESEM image of *Drosophila virilis* eggshell

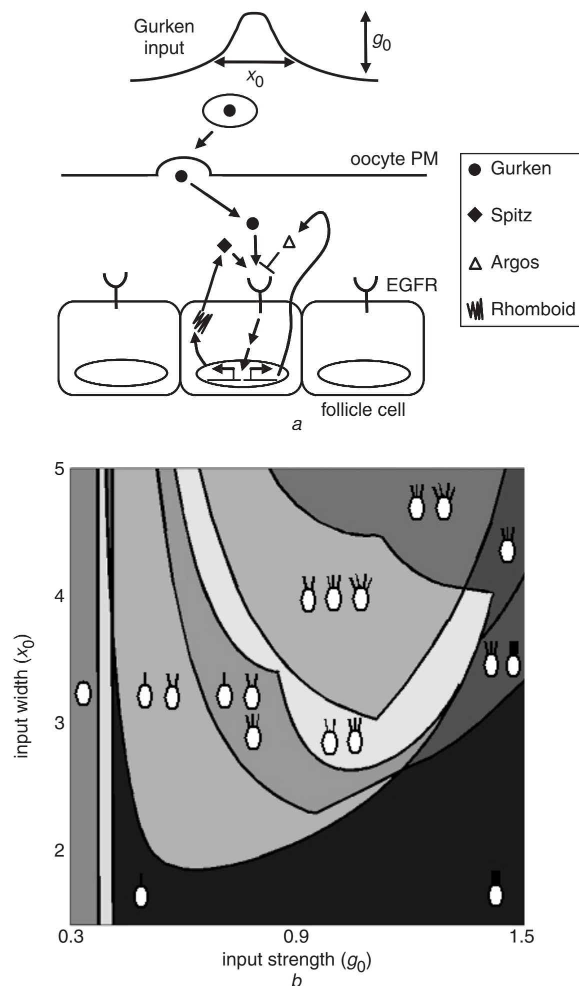

Fig. 4 *Model of pattern formation*

a Input and feedback loops in model of pattern formation by peak splitting
b Summary of results of computational analysis of one-dimensional model of pattern formation by *Drosophila* EGFR autocrine feedback loops
Regions of existence of different stationary patterns as function of width (x_0) and amplitude (g_0) of input (Gurken) signal. Patterns with different number of peaks are associated with eggshells with different number of dorsal appendages (shown by insets). See [32] for detailed definition of model parameters and its computational analysis

model, mimicking the oocyte-derived Gurken, can generate a stable pattern with two large-amplitude peaks in the spatial distribution of Rhomboid. The two-peaked patterns emerge as a result of the instability of the one-peaked solution that is realised at lower inputs. At a critical input level, this single-peaked solution splits, giving rise to the blueprint for the formation of two dorsal appendages. Thus, within the framework of this model, patterning leading to the formation of dorsal appendages can be viewed as a transition between the two kinds of solution in the model (i.e. one- and two-peaked). Specifically, the model predicted that increased levels of the oocyte-derived Gurken signal can generate eggshells with three or four dorsal appendages, similar to the wild-type eggshells of *D. phalerata* and *D. virilis* [28–30]. We have tested this prediction experimentally and have indeed recovered eggshells with three and four dorsal appendages. As such phenotypes cannot be generated within the gradient-interpretation model, this experiment seems to favour the feedback model. At the same time, the gradient model predicts continuous separation between appendages in response to increased Gurken level, whereas the feedback model cannot account for this experimental observation [27, 32].

Recently, the biochemical mechanism of EGFR inhibition by Argos has been firmly established [20]. As it turned out, Argos inhibits EGFR by sequestering its activating ligand Spitz and not by interacting with the receptor itself. This biochemical information is yet to be incorporated into the mathematical model of follicle cell patterning. Although both the gradient interpretation and the feedback mechanisms are EGFR-centric, rapidly accumulating evidence suggests that another pathway plays an important role in cell fate induction in the follicular epithelium. Specifically, signalling through serine-threonine kinase receptors, homologous to the mammalian bone morphogenetic protein (BMP) pathway, is essential for the induction of dorsal appendages and operculum [14, 34]. As the BMP signalling is understood much less than signalling through receptor tyrosine kinases, modelling of these processes at this time can be phenomenological at best.

6 Systems-level questions in *Drosophila* oogenesis

Although it is safe to say that a detailed mathematical model of follicle cell patterning will take some time to establish, we emphasise that already now any model can be sufficiently constrained by a large number of experimental data from egg chambers with altered EGFR and/or BMP signalling. In addition, genetic perturbations in combination with high-throughput transcriptional profiling experiments can be used to construct increasingly accurate molecular descriptions of *Drosophila* oogenesis. At this stage, our work focuses on the properties of, and response to, the Gurken signal. Specifically, we consider the following issues:

(*a*) The shape and the time course of the Gurken signal: The Gurken signal is essential for patterning of the eggshell, but this gradient cannot be visualised and can be perceived only indirectly, through its effects on signalling and transcriptional targets of EGFR. By following how these targets respond to perturbations of the EGFR system in oogenesis, it might be possible to infer the shape and the dynamics of the Gurken input to the follicle cells.

(*b*) The genes responding to the Gurken signal: We know ~20 genes responsive to Gurken, but it is important to characterise response to the Gurken gradient at the scale of the whole genome. It is clearly of interest to determine just how many genes are involved in patterning of the eggshell. When the list of the genes responding to the Gurken signal is established, it will be important to investigate how the Gurken signal ‘selects’ these genes from the entire genome.

(*c*) Pathways involved in patterning of the follicle cells: So far, the EGFR and BMP receptor pathways have been identified as critical regulators of follicle cell patterning and eggshell morphogenesis, but it is necessary to consider whether other pathways are involved and how they interact with already established players. At this time, the patterns of co-ordinate regulation of genes by multiple extracellular signals are very poorly understood.

With a proper ‘change of variables’, these issues can be considered for many of the other experimental systems in which complex patterns of cell fates are established in the layer of initially identical cells [1]. Below, we outline the experimental approaches that can be used to address these issues in *Drosophila* oogenesis.

7 Experimental perturbations of *Drosophila* oogenesis

EGFR signalling in *Drosophila* oogenesis can be genetically perturbed in a variety of ways, and the effects of perturbations can be monitored at multiple levels, from eggshell morphology, to patterns of expression of individual genes, to co-ordinate response of multiple genes [9]. For example, using the techniques for tissue-specific gene expression [35], it is possible to perturb the EGFR network in the follicle cells by expressing any one of the three induced intracellular inhibitors (Argos, Kekkon and Sprouty), or the dominant negative form of EGFR (dn-EGFR); see Fig. 5. All of these perturbations lead to ventralised eggshells (with reduced or missing operculum and either fused or missing dorsal appendages), with the strongest effect induced by dn-EGFR; see Fig. 5*f*. At the same time, expression of a constitutively active form of EGFR (ca-EGFR) dorsalises the eggshell; see Fig. 5*b*: the operculum cell fate is expanded at the expense of dorsal appendages and ventral cell fates.

In any one of these genetic backgrounds, *in situ* hybridisation assays can be used to visualise the expression of the known targets of EGFR signalling in oogenesis. For example, the spatial domain of expression of *pip*, a gene repressed by EGFR [11, 16], expands in the egg chambers with reduced EGFR signalling and is completely abolished by over-expression of ca-EGFR; see Fig. 6. These changes in the expression patterns of known targets of EGFR correlate well with the corresponding eggshell morphologies.

Whereas *in situ* hybridisation assays usually focus on only one or two genes, quantitative real-time PCR (qRT-PCR) and microarray-based assays can monitor multiple genes at the same time, albeit without the spatial resolution provided by *in situ* hybridisation. For example, qRT-PCR can quantify the expression of multiple genes across stages of oogenesis in multiple genetic backgrounds. An example of such an experiment is shown in Fig. 7, where we compare the levels of expression of intracellular inhibitors of EGFR in egg chambers that lack the oocyte-derived Gurken signal, express the constitutively active form of the receptor, or express a dominant negative form of the receptor. Finally, microarray-based approaches can monitor the genome wide transcriptional responses to a variety of genetic perturbations [36]. Statistical and bioinformatics

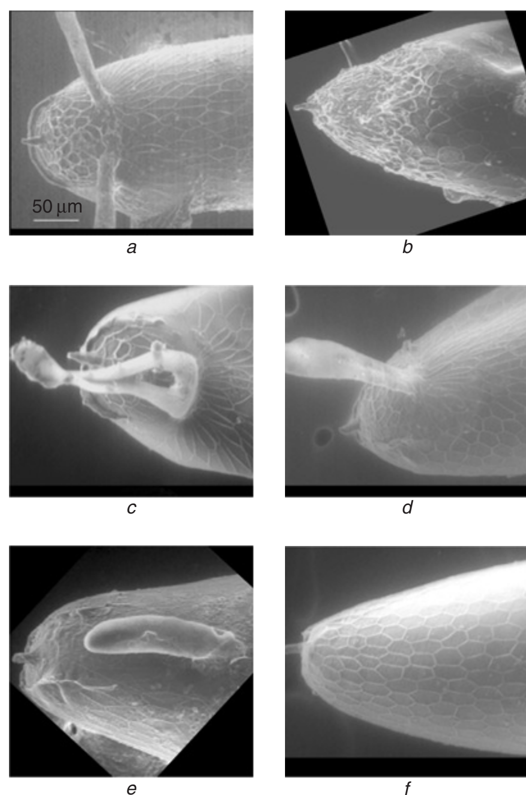

Fig. 5 *ESEM images of dorsal-anterior part of eggshells derived from egg chambers with genetically perturbed EGFR signalling*

a Wild type
b Constitutively active form of EGFR
Effects of EGFR inhibitor expression:
c Argos
d Sprouty
e Kekkon1
f Effect of overexpression of dominant negative EGFR
Each perturbation was generated by expressing construct uniformly throughout follicular epithelium using Gal4/UAS system for tissue specific gene expression

analyses of the data generated by transcriptional profiling experiments can be used to discover new genes regulated by EGFR and stimulate further experiments on EGFR-mediated pattern formation in oogenesis and other stages of *Drosophila* development.

8 Data integration

The described experimental approaches to pattern formation in *Drosophila* oogenesis are generating a rapidly increasing number of heterogeneous data that include three-dimensional images of mature eggshells, overall transcriptional responses from qRT-PCR and microarray assays, and spatial patterns of gene and protein expression. As the number of analysed genetic backgrounds increases, it will be more and more difficult to remember how a particular gene responded to a list of genetic perturbations or to generate lists of similarly expressed genes. At this level of data complexity, it is becoming essential to develop web-based tools for effective submission, storage, tracking, processing, visualisation and querying of these data.

As a first step in this direction, we have developed a platform for the automated processing, statistical analysis, storage and display of qRT-PCR and microarray data; see Fig. 8*a*. Integrating the newly generated datasets with the wealth of existing biological knowledge from published papers and large-scale datasets is crucial for system-level analysis; Fig. 8*b*. This integration highlights the need to

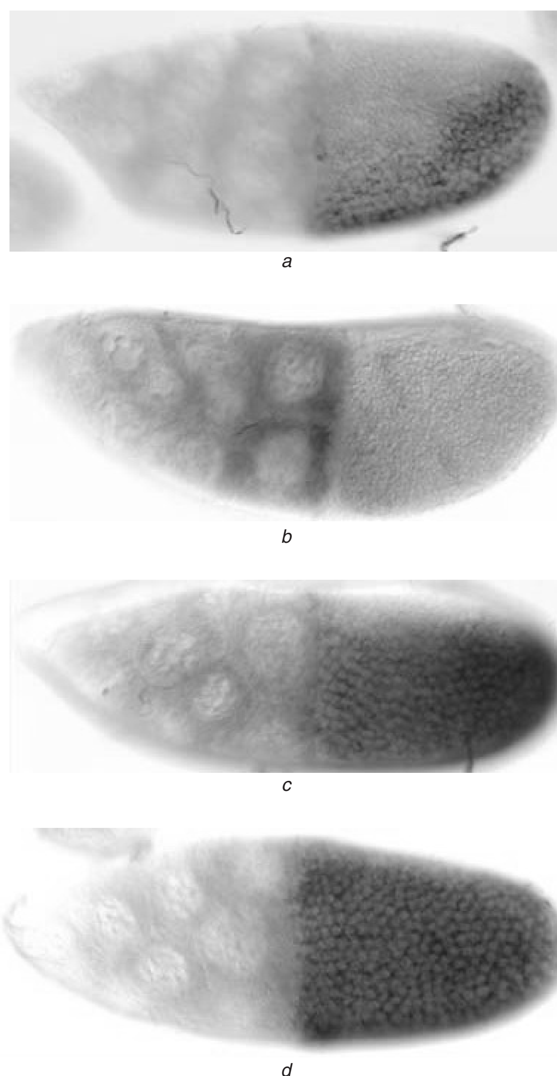

Fig. 6 *Effects of genetic perturbations of EGFR signalling on spatial pattern of pip, one of transcriptional targets of EGFR signalling in follicular epithelium*

a Pip expression in wild-type egg chamber spans 30–40% of ventral domain of egg chamber
b Uniform expression of constitutively active form of EGFR eliminates ventral domain of *pip*
c Uniform expression of intracellular inhibitor of EGFR signalling (Sprouty) expands domain of *pip*
d When dominant negative form of EGFR is expressed uniformly across follicular epithelium, *pip* domain spans all follicle cells associated with oocyte

use common annotations, such as Celera gene numbers and Flybase identifiers. We are currently developing systems for the storage and display of the steadily increasing numbers of images of spatial patterns of gene expression and morphological features in *Drosophila* oogenesis. To facilitate higher-level queries, we must extend and formalise the pre-existing *Drosophila* oogenesis vocabulary (e.g. ‘ventralised’ or ‘expanded operculum’) to describe the observed phenotypes. Similar efforts are currently underway for other stages of fruit fly development [37–39].

9 Towards mechanistic modelling

As our understanding of the eggshell patterning network progresses, it will be possible to formulate fully mechanistic models that should predict the dynamics of, and responses to, the morphogen gradients that pattern the follicular epithelium. As the spatial patterns of gene expression in the

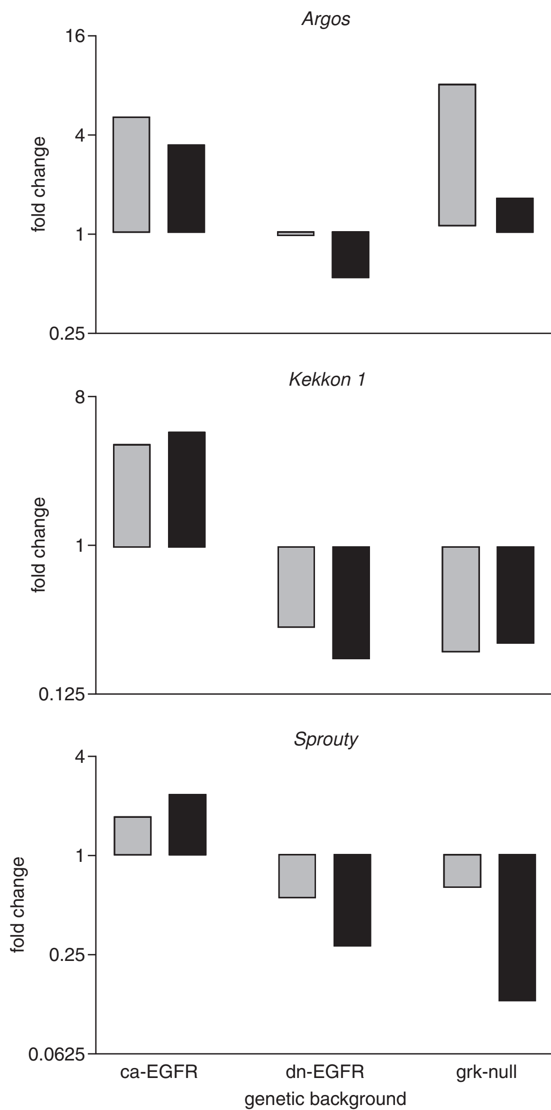

Fig. 7 *Transcriptional profiling of three EGFR-induced inhibitors of EGFR signalling (Argos, Kekkon1, Sprouty) in follicular epithelium*

We have used both qRT-PCR (grey bars) and Affymetrix gene chips (black bars) to monitor level of expression of each of three inhibitors in three genetic backgrounds corresponding to overexpression of constitutively active form of EGFR (ca-EGFR), dominant negative form of EGFR (dn-EGFR) or lack of oocyte-derived Gurken signal (grk-null)

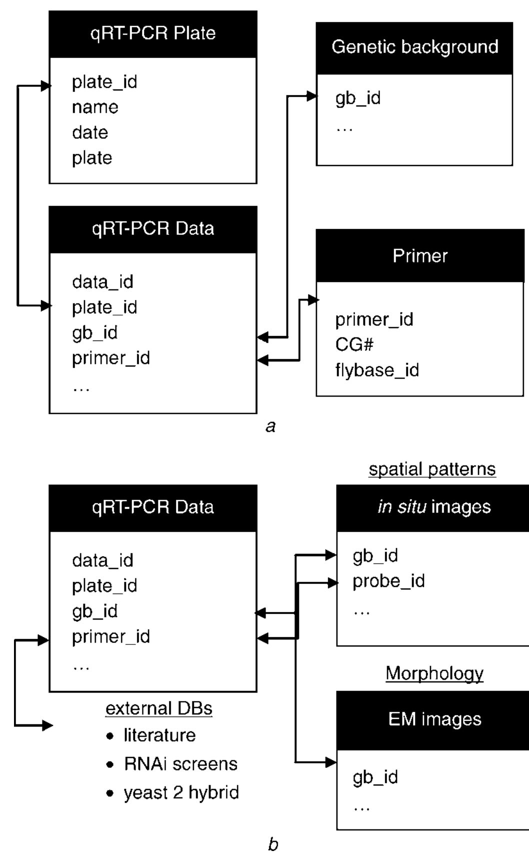

Fig. 8 *Organisation of qRT-PCR data*

a Web-based interfaces are used to facilitate automated analysis, storage and display of qRT-PCR data. Overall organisation relies on four data models describing assay conditions, reaction in each well, primers available and genetic backgrounds. Using this system operator annotates each qRT-PCR well with unique tag and submits raw data to computational pipeline. Data are normalised to loading control, and fold change is calculated using delta–delta ct method, and statistical significance is tested using a student's *t*-test. Raw and processed data are stored in database, and web-based interfaces allow for queries to be preformed

b Integration of qRT-PCR data with spatial patterns of gene expression and morphological features relies on joining data models using genetic background and primer indexing system. Use of common annotations such as CG #s and Flybase IDs are used to integrate heterogeneous datasets

follicular epithelium can be fine grained (e.g. the stripes in the *rho* pattern are just a single cell wide), these models must account for the 'discrete' nature of the patterned cellular layer. Our first approaches to this class of problems have produced an interesting class of hybrid (continuous/discrete) dynamic systems with solutions that might differ from solutions of fully continuum reaction-diffusion equations [40, 41]. For example, we have developed mechanistic models for the Rhomboid/Spitz circuit that amplifies the oocyte-derived Gurken signal in eggshell patterning. The EGFR-activated Ras/MAPK pathway, together with the BMP pathway, induces Rhomboid, which then stimulates the secretion of Spitz that binds to EGFR on the ligand-producing cells and their neighbours. This information about signalling in a single cell can be combined with the ligand transport model for analysis of the operation of the Rhomboid/Spitz feedback in a multicellular system such as an epithelial layer. The resulting description is useful in analysing the effects of exogenous signals presented to the epithelial layer. To describe the operation of the Rhomboid/Spitz circuit, we developed models of autocrine signalling in epithelial layers. In addition to ligand transport, binding and internalisation, these models account for Rhomboid induction and Rhomboid-mediated Spitz release. Rhomboid induction was modelled as a threshold-like function σ of the total number of ligand-receptor complexes on the cell surface. The balance for the level of Rhomboid in the cell (i, j), $P_{i,j}$, takes the following form:

$$\frac{dP_{i,j}}{dt} = -\frac{P_{i,j}}{\tau} + \sigma(C_{i,j}^{tot} - C_T)$$

where $C_{i,j}^{tot}$ is the total number of occupied EGF receptors in the cell (i, j), and C_T is the threshold-value for Rhomboid induction. Receptor occupancy on any given cell within the epithelial layer depends on the pattern of ligand release and, hence, the pattern of Rhomboid expression in the entire layer. Our analysis suggests that ligand binding and transport rapidly adjust to the much slower dynamics of Rhomboid expression. In other words, the equations for ligand binding and transport reach the steady state dictated

by the pattern of Rhomboid across the epithelial layer. In the ligand-limited regime, receptor occupancy for a given cell is computed from the linear superposition of ligand fields due to individual cells. As a result, the dynamics of cells coupled by secreted signals can be described entirely in terms of the intracellular variables

$$\frac{dP_{i,j}}{dt} = -\frac{P_{i,j}}{\tau} + \sigma\left(\sum_{m,n} I_{|i-m|,|j-n|} P_{m,n} - C_T\right)$$

where $I_{|i-m|,|j-n|}$ is the cell–cell coupling coefficient that quantifies the strength and the spatial range of autocrine and paracrine signals. Importantly, these coefficients were derived as a function of the biophysical parameters of the problem, such as the diffusion and binding rates, as well as the rates and the levels of ligand release by single cells within the layer. We found that the coupling coefficients decay rapidly as a function of cell–cell distance. This suggests that only a small number of cell–cell interactions must be taken into account in calculating receptor occupancy on any given cell, a fact that is very useful in solving the problem numerically. This biophysical framework can be used to predict the possible effects of localised perturbations of epithelial layers. We note that, although our long-term goal is to use these models for the analysis of particular biological pattern formation problems, the development of appropriate analytical and numerical methods for the analysis of these models is interesting and challenging in itself.

10 Materials and methods

10.1 Fly stocks

The following fly strains were used: wild type starins *Drosophila melanogaster* (*Ore*R), *D. phalerata*, *D. virlis.* Additional strains were: *grk*^{HF} cn bw (grk null) [42], *UAS-λ-top* 4.2 (ca-EGFR) [43], UAS-sprouty, UAS-kekkon1 and UAS-agros (a gift from Joseph Duffy), and UAS-dn-EGFR (a gift from Alan Michelson). All UAS lines were driven by *CY2-gal4*, line as described in [43].

10.2 Electron microscopy imaging

Contrary to traditional scanning electron microscopy (SEM), where a sample needs to be dry and conductive to be observed, environmental scanning electron microscopy (ESEM) allows for imaging of biological and insulating samples without the need for coating [44]. In the case of the ESEM, water vapour is circulated through the imaging chamber at pressures of up to 20 Torr (760 Torr = 1 atm). Through interaction with the electron beam, the gas molecules become ionised and attracted to the charged surface of an insulating sample, neutralising it. The ESEM was used in this study to image the *Drosophila* eggshells in their natural state at high magnification.

10.3 Dissection protocol and RNA extraction

All flies were grown at 23°C on standard media. Bakers yeast was added to the fly food 24–48 h before dissection. Ovaries were hand dissected in cold 1 X PBS, and developmental staging separation was carried out according to Spradling [7]. Egg chambers from stages 9 and 10 were separated from the ovariole and put in RNA stabilising buffer[1]

[1]RNeasy Mini Kit, Qiagen, Valencia, CA

Total RNA was extracted according to manufacturer instructions from approximately 100 egg chambers. RNA qualification was performed in a Gene Chip RNA 6000 Nano Assay[2] RNA quantification was performed on 1 μl total RNA sample in a NanoDrop ND-1000 spectrophotometer[3].

10.4 Quantitive real time PCR, Affymetrix gene chips and data analysis

Three independent total RNA samples (biological replicates) were prepared for each of the three experimental conditions and the wild type (*Ore*R, *grk*^{HF}, *ca-EGFR*, *dn-EGFR*). Each of the samples was analysed twice, resulting in two technical replicates. Statistical analysis was performed on the groups of three biological replicates with averaging over two technical replicates. 1 μg of each of the total RNA samples was used for first strand cDNA synthesis using TaqMan Reverse Transcription Kit,[4] according to the manufacturer's protocol. For qRT-PCR, the reaction consisted of calculated 25 ng first strand cDNA template, primer mix, ROX and SYBR Green PCR mix,[5] in a total volume of 25 μl. The average fold change of the target genes in mutant genetic backgrounds relative to wild type (*grk*^{HF}/*Ore*R, *ca-EGFR*/*Ore*R and *dn-EGFR*/*Ore*R) and normalised to *rp*49 expression were calculated using the $2^{(-\Delta\Delta C_t)}$ method [45]. A Student's two-tailed *t*-test was used to determine if the means of the biological replicates of each mutant background, compared with the wild type, were statistically significant ($p \leq 0.05$). The qRT-PCR experiments were performed using MX-3000P (Stratagene). To validate the specificity of the PCR reaction, a melting curve was produced by PCR end product denaturation from 55 to 95°C at 0.5°C 30 s^{-1}.

Biotinylated cRNA was generated from total mRNA isolated from the four genetic backgrounds using the one-cycle target labelling and control reagents (Affymetrix). Three independent samples for each background were hybridised to Affymetrix *Drosophila* arrays (DrosGenome1) using standard Affymetrix protocol. The data analysis options for background correction, probe level normalisation, perfect match adjustment, expression summary, probe set normalisation and statistical analysis were conducted as by the 'Golden Spike' recommended algorithms [46].

10.5 In situ hybridisation and immunoassay

For *in situ* hybridisation, ovaries dissected in 1 X PBS were fixed in 4% paraformaldehyde in 1 X PBS + 10% DMSO + 0.1% Tween 20 and three volumes of *n*-heptane for 20 min. Thereafter, the solution was changed to 4% paraformaldehyde in 1 X PBS + 0.1% Tween 20 for 5 min, followed by 90% methanol + 10% 50 mM EGTA. Fixed ovaries could be stored in 100% methanol at −20°C. cDNA plasmids for *pip* were provided by the D. Stein, and the digoxigenin-labelled antisense RNA probe was prepared with Roche reagents. For Broad immunoassay, ovaries were fixed in 4% paraformaldehyde in 1 X PBS with 0.1% Tween 20 for 20 min and stored in methanol at −20°C. The remaining protocol steps were performed as previously described [47], using the Elite Vectastain Kit.[6]

[2]Agilent Technologies, Palo Alto, CA
[3]NanoDrop Technologies, Wilmington, DE
[4]Roche, Branchburg, NJ
[5]Stratagene, La Jolla, CA
[6]Vector Laboratories, Inc. Burlingame, CA

10.6 Database development

The range of experimental data and their attributes were stored using the MySQL relational database management system. Web-based interfaces for submission, processing and display were written in Perl using CGI.pm, DBI.pm and GD.pm Perl modules.

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