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Understanding morphogen gradients: a problem of dispersion and containment

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Protein morphogens are instructive signals that regulate growth and patterning of tissues and organs. They form long-range, dynamic gradients by moving from regions of high concentration (producing cells) to regions of low concentration (the adjacent, nonproducing developmental field). Since morphogen activity must be limited to the adjacent target field, we want to understand both how signaling proteins move and how their dispersion is restricted. We consider the variety of settings for long-range morphogen systems in *Drosophila*. In the early embryo, morphogens appear to disperse by free diffusion, and impermeable membranes physically constrain them. However, at later stages, containment is achieved without physical barriers. We argue that in the absence of constraining barriers, gradient-generating dispersion of morphogens cannot be achieved by passive diffusion and that other mechanisms for distribution must be considered.

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Current Opinion in Genetics & Development 2007, **17**:264–271

This review comes from a themed issue on
Pattern formation and developmental mechanisms
Edited by Ross Cagan and Christine Hartmann

Available online 23rd July 2007

0959-437X/\$ – see front matter
Published by Elsevier Ltd.

DOI [10.1016/j.gde.2007.05.010](https://doi.org/10.1016/j.gde.2007.05.010)

Introduction

Concentration gradients of protein morphogens are thought to embody the informational landscapes that regulate and pattern developmental fields. As a function of either absolute or relative concentration, they elicit differential responses across a field of target cells. Most of these gradients form by dispersion from a localized source, and their activity is transmitted through widely distributed receptors. Although morphogen movement has been studied intensively and has been the subject of numerous excellent reviews (recent ones include [1,2–7]), neither experimental nor theoretical analysis has established the mechanisms that distribute these proteins across developmental fields. This review focuses on an aspect of morphogen gradients that has received little attention — the means by which morphogen movement is limited. It

examines morphogen gradients in the *Drosophila* oocyte, precellular embryo, and wing imaginal disc, and argues that the architecture of the oocyte, embryo, and disc bears directly on the manner by which morphogens are restricted to their intended targets. These considerations have significant implications for the possible mechanisms that disperse morphogens in these different settings.

Gradient systems of the *Drosophila* oocyte and embryo

Several aspects of the morphogen gradients that organize the *Drosophila* oocyte and embryo are unique. First, the proteins that form instructive concentration gradients at these developmental stages are not employed again as morphogens at later developmental stages. Second, the concentration gradients signal in three-dimensional space, either from one cell layer to another or across the volume of the embryo. Third, although their concentration profiles are controlled in part by kinetics of synthesis, activation and degradation, their dispersion is apparently unhindered except that impermeable barriers define the limits of their movement. In this review we distinguish between the outcome of morphogen movement (distribution/dispersion across a target field) and mechanism of movement (diffusion is one means of dispersion). Known properties of these proteins are consistent with passive diffusion as the most probable mechanism for distributing them into concentration gradients.

Oocyte gradients

Multiple gradient systems set up the anteroposterior and dorsoventral polarity axes of the early *Drosophila* embryo. The first to act in the developing oocyte generate regional specialization among the overlying somatic follicle cells (Figure 1a, reviewed in [8]). Follicle cell specialization is directed by Gurken, which is secreted from distinct regions of the oocyte and is a ligand for the EGF receptor. After fertilization, discrete populations of specialized follicle cells direct the formation of separate gradients that specify the embryo anteroposterior and dorsoventral axes. In early oogenesis, Gurken is produced near the posterior of the egg chamber, and secreted there, it engages its receptor in the adjacent follicular epithelium to specify posterior follicle cell fate. As the oocyte grows, a second Gurken signal emanates from the anterodorsal portion of the oocyte; this second signal generates distinct dorsoventral fates among the follicle cells. Neither the Gurken nor the EGFR gradients have been observed directly. They are inferred from the expression of downstream targets of the EGFR signal transduction pathway

[9]; they are believed to form in the space between the oocyte plasma membrane and the surrounding follicular epithelium — more importantly, the space in which Gurken signals is closed. Its boundary membranes limit dispersion and insulate surrounding tissue layers from its resident signaling proteins.

Anteroposterior gradients in the embryo

After fertilization, two intracellular gradient systems organize the anteroposterior axis of the precellular embryo by distributing concentration gradients of the nucleic acid binding proteins, Bicoid and Nanos. A gradient forms when Bicoid is synthesized at the anterior pole of the embryo and disperses within the syncytial cytoplasm (Figure 1b, reviewed in [10]). A similar mechanism organizes the posterior regions, where a concentration gradient of the Nanos protein forms. Both protein gradients have been observed directly by staining early embryos with antibodies. At these early stages of embryogenesis, cell membranes have not formed around the rapidly dividing nuclei that populate the syncytial embryo; the embryo is a multinucleated single cell, and Bicoid and Nanos are apparently constrained only by the embryo plasma membrane. Studies of how inert probes spread in the syncytial embryo and of the temporal properties of the Bicoid gradient are consistent with a diffusion model [11^{••}], so it seems reasonable to conclude that the Bicoid and Nanos proteins spread freely through the embryo from their respective sources.

Embryo terminal gradients

The gradient systems that pattern the anterior and posterior termini are thought to form in the fluid-filled space between the precellular embryo and its encapsulating vitelline membrane. The systems are similarly constituted, employing the Torso transmembrane receptor that is uniformly distributed in the embryo plasma membrane (Figure 1c; [12]). Localized activation at each pole by the Trunk ligand leads to downstream induction of signal transduction pathways. Trunk has been detected in the perivitelline fluid and the generally accepted model proposes that Trunk distributes uniformly in the perivitelline fluid as an inactive pro-protein, but that after localized proteolytic activation initiated by follicle cells at each pole, active Trunk diffuses in the perivitelline fluid to bind and activate its receptors embedded in the plasma membrane of the embryo. As with Gurken, the Trunk gradients have not been observed directly, nor have the dispersive properties of Trunk in the perivitelline fluid been examined; the gradients are presumed to exist because of the graded patterns in which the downstream targets of the signal transduction pathways are activated. Importantly, the dispersion of Trunk is constrained. Dispersion is limited on the outside by the vitelline membrane and its waxy coat, and on the inside by the embryo plasma membrane. These membranes define a narrow space within which the presumed gradients form.

Embryo dorsoventral gradient

The gradient system that specifies the dorsoventral axis of the embryo is mechanistically similar to the terminal gradients, employing a uniformly distributed transmembrane receptor (Toll) in the embryo and a uniformly distributed inactive pro-protein ligand (Spätzle) in the perivitelline fluid. Spätzle activation is thought to be initiated by specialized follicle cells located along the ventral midline, and activated Spätzle is thought to diffuse in the perivitelline fluid (Figure 1d, [13]). The resulting gradient of Toll signal transduction is in turn reflected in a gradient of nuclear translocation of the Dorsal transcription factor. Dorsal regulates numerous target genes in a concentration-dependent manner in the presumptive mesoderm and neuroectoderm, including *decapentaplegic* (*dpp*).

Gradient systems of the *Drosophila* wing imaginal disc

The morphogens that pattern organ systems during embryogenesis and larval development operate in settings that differ from those of the precellular *Drosophila* embryo. We will focus this discussion on the wing primordium of the wing imaginal disc. The protein morphogens that regulate the wing primordium appear to play similar roles in many different vertebrate and invertebrate organs, and if we assume that the mechanisms that disperse these morphogens are also conserved in these other settings, then the physical attributes of the wing primordium that affect dispersion are relevant to other systems as well. More importantly, the wing disc lacks physical barriers that insulate closely juxtaposed developmental fields.

Wing primordium patterning systems

Three systems pattern the wing primordium. These are embodied by Hedgehog (Hh), Dpp, and Wingless (Wg) protein gradients (see Figure 2a). Wg and Dpp are produced along the D/V and A/P compartment borders, respectively; both proteins disperse from a band of expressing cells at the respective borders to generate concentration gradients. Hh is produced by all P compartment cells; it moves across the A/P compartment border, decreasing in concentration with distance from the border. The Hh, Dpp, and Wg receptors are transmembrane proteins, and Hh, Dpp, and Wg are secreted proteins.

Physical parameters of the wing disc

Development of the wing disc starts when approximately 10–24 cells in the second thoracic segment that straddle the anteroposterior compartment border invaginate from the embryo epithelium [14]. By the end of the third larval instar, a program of cell division and morphogenesis generates a flattened sac that has two distinct surfaces and that remains connected to the larval epidermis by a proximal stalk. Squamous peripodial cells populate one surface of the disc, columnar epithelial cells are on the

Figure 1

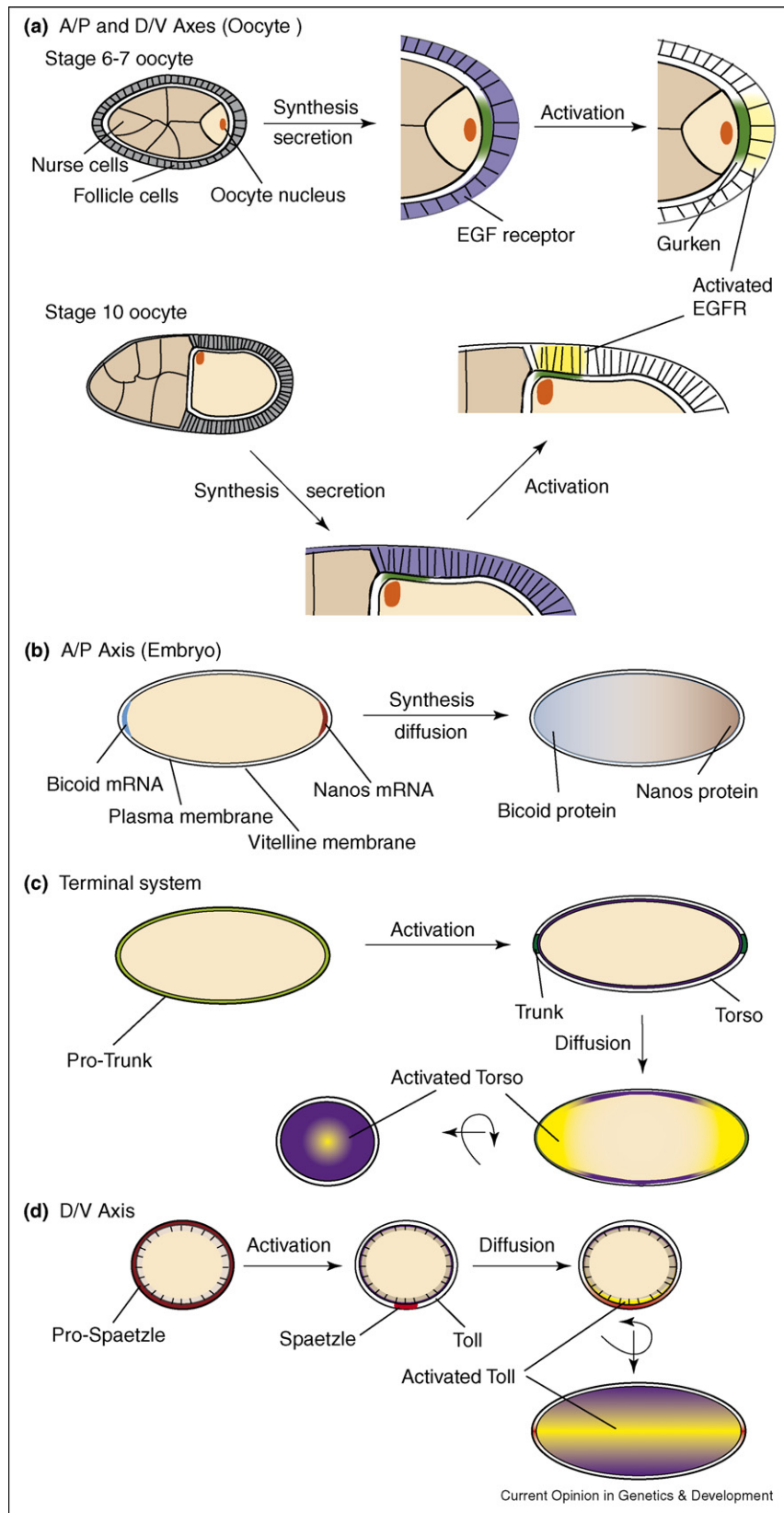
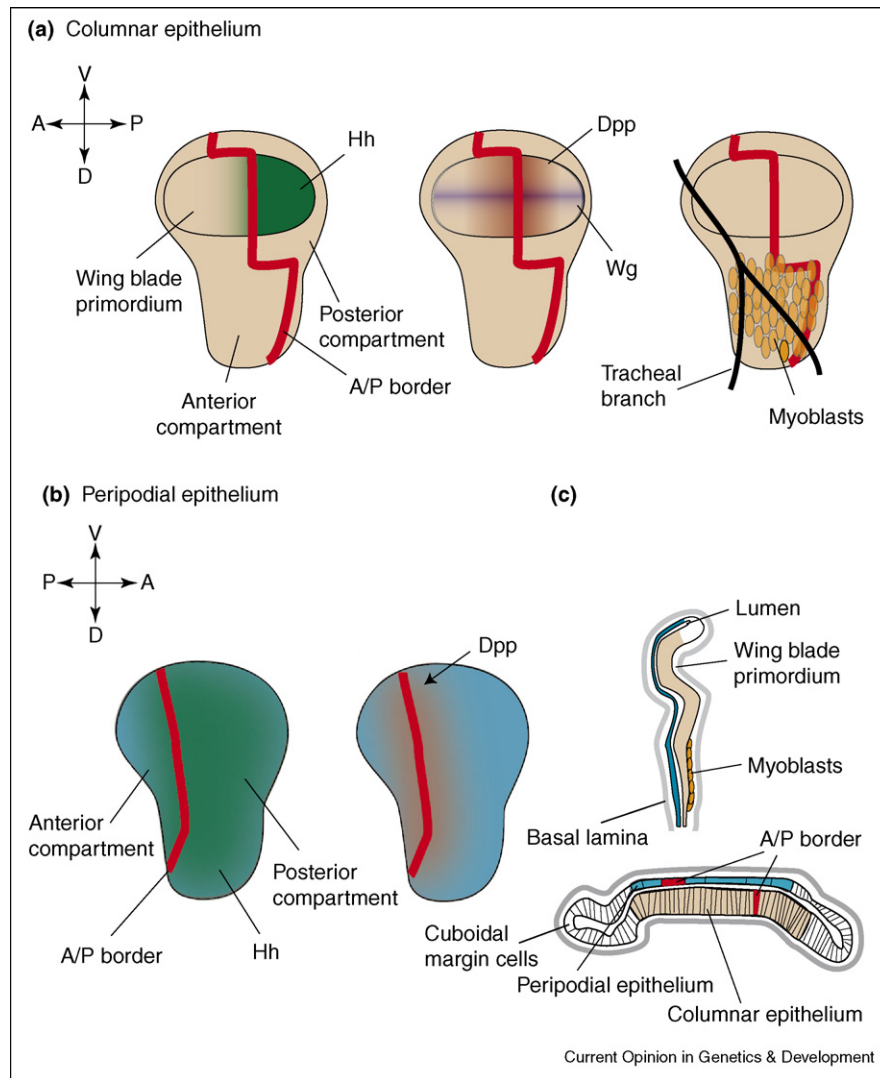


Figure 2



Organization of the wing imaginal disc. A third instar wing disc viewed from the (a) columnar epithelium side (tan), (b) peripodial epithelium side (blue), and (c) in cross-section. The A/P border (red) is contiguous on both surfaces. Hh (green) is expressed in all P compartment cells. Distributions of Hh, Dpp (ruby) and Wg (purple) in the wing primordium and peripodial epithelium, the disc-associated tracheal branch and myoblasts (orange) are shown in (a).

other, and cuboidal margin cells connect the two surfaces (Figure 2). The peripodial and columnar surfaces in the region of the wing blade primordium are separated by only 6 μm [15]. Despite the close juxtaposition of the two

surfaces, the anteroposterior compartment border, which runs contiguously along the two surfaces, is not aligned (Figures 2b, c, and 3b). This failure to align is relevant to morphogen dispersion and containment, since compart-

(Figure 1 Legend) Morphogen gradient systems in the *Drosophila* oocyte and early embryo. (a) A/P and D/V axes (oocyte) — definition of the anteroposterior and dorsoventral axes of the embryo that initiates during oogenesis when *gurken* is expressed by the posteriorly situated oocyte nucleus (orange) at stage 6–7. At this stage, the oocyte is relatively small and is juxtaposed to nurse cells (brown) and to somatic follicle cells (gray). All follicle cells express the EGFR Gurken receptor (purple), but the posterior follicle cells closest to oocyte nucleus presumably receive most of the secreted Gurken protein (green) and are activated (yellow). At stage 10, the oocyte nucleus has assumed an anterodorsal position, and upon expression of *gurken*, EGFR activation induces dorsal cell fates among the nearby follicle cells. (b) A/P axis (embryo) — postfertilization, *bicoid* (blue) RNA and *nanos* RNA (red) sequestered at the A and P poles, respectively, are translated; Bicoid and Nanos proteins disperse across the syncytium. (c) Terminal system — inactive pro-Trunk (lime green) and the Torso receptor (purple) are distributed uniformly in the perivitelline fluid and embryo plasma membrane, respectively. Following proteolytic activation initiated by follicle cells at the A and P poles, active Trunk (dark green) disperses and activates Torso (yellow). (d) D/V axis — inactive pro-Spaetzle (burgundy) and the Toll (purple) are distributed uniformly in the perivitelline fluid and embryo plasma membrane, respectively. Following proteolytic activation initiated by follicle cells along the ventral midline, active Spaetzle (red) disperses and activates Toll (yellow).

ment borders on both surfaces are associated with organizing centers that are sources of morphogens.

In addition to the stalk, peripodial, columnar and margin cells, the wing disc also has intimately associated myoblasts and tracheal cells. The myoblasts are the precursors of the adult thoracic muscles; the tracheal cells populate tracheal branches that adhere to the disc but do not appear to serve it with oxygen. All of the myoblasts and portions of the tracheal branches lie within the basal lamina that lines the outer, basal surface of the disc (Figure 2a and c).

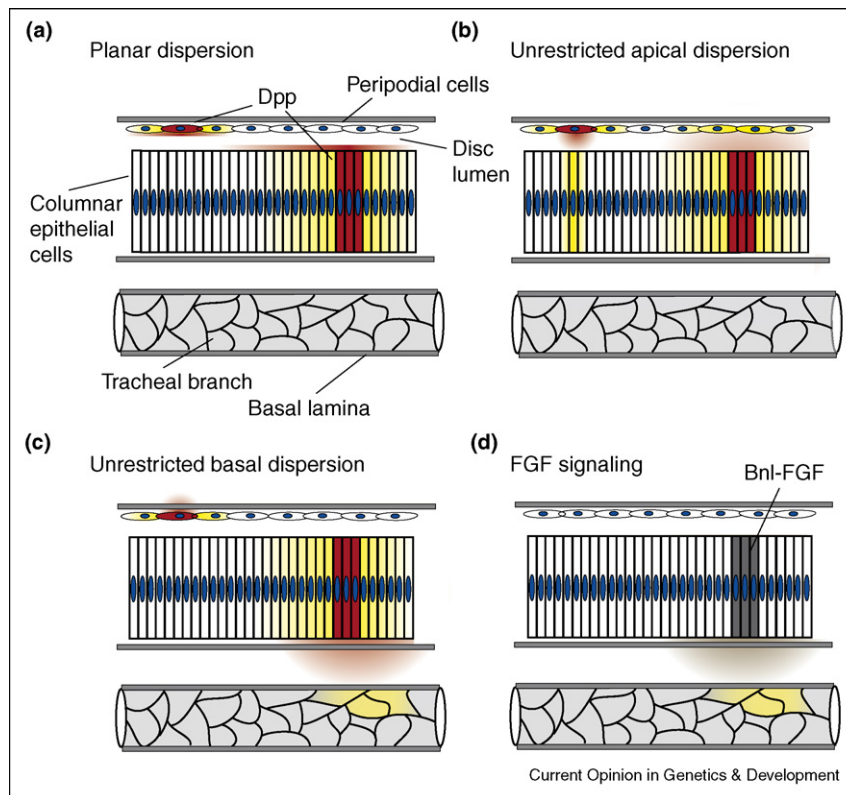
Signaling parameters of the wing disc

The distance that Dpp moves from its site of synthesis at the anteroposterior compartment border to the edge of the wing primordium, approximately 100 μm , is much greater than the 6 μm that separates the peripodial and columnar layers. Peripodial cells require both Hh and Dpp for normal morphogenesis [16], so the close proximity of the columnar and peripodial cell layers in wing discs raises the possibility of direct cross talk between the layers. We assume that morphogens expressed by any of

the signaling centers do not affect cells across the disc lumen, despite the close proximity. Cross-luminal signaling between these cell layers has been observed upon ectopic overexpression of Hh in the peripodial epithelium [17^{••}], and these discs do not develop normally. We interpret the phenotypes described by Gallet *et al.* (2006) to indicate that the capacity of the system to limit signaling to the plane of an epithelium can be overridden (for instance by deleting the cholesterol modification of Hh) or can be overwhelmed (for instance, by overexpression of both Hh and dispatched). Importantly, no impermeable barrier prevents cross-luminal signaling.

Hh, Dpp, and Wg have been detected in the lumen of the wing disc [17^{••},18,19], but these studies do not reveal how these proteins distribute within the lumen. They do not resolve whether the concentration of the proteins is uniform in the lumen or whether the proteins localize to the apical surface of the epithelium. Since we assume that the normal, wild-type condition does not permit unrestricted cross-luminal signaling, we propose that the proteins do not move far from the epithelial surface

Figure 3



Models of Dpp and Bnl-FGF dispersion in wing discs. Columnar and peripodial cells are depicted in cross-section, as is the disc-associated tracheal branch. Basal lamina encapsulates both disc and tracheal branch (gray). Dpp (red) is expressed at the developmental organizers/signaling centers of the columnar and peripodial layers (a–c); Bnl-FGF (black) is produced by columnar epithelial cells (d). Activation (yellow) is depicted in both disc and tracheal cells. (a) If Dpp is restricted to the epithelium surface, its activity is predicted to be restricted. (b) If Dpp moves freely after apical secretion, cross-luminal signaling is predicted. (c) If Dpp moves freely after basal secretion, it is predicted to activate both disc and tracheal cells, since basally secreted Bnl-FGF has been shown to signal through the basal lamina (d).

(Figure 3a). This argument does not preclude cross-luminal communication that is unrelated to morphogen gradients [18], but suggests that if it occurs, it does so separately from the mechanisms that generate the gradients.

Studies of FGF signaling have provided an informative example of cross talk between distinct cell layers in the wing disc. Disc cells do not express the FGF receptor, *Breathless*, and do not respond to Bnl-FGF. However, ectopic expression of Bnl-FGF by wing disc cells induces nearby tracheal branches to generate ectopic outgrowths (A Guha, TB Kornberg, unpublished; [20]). The responsiveness of the tracheal cells indicates that signaling between the disc and tracheal cells occurs despite the presence of interposed basal lamina. Many vertebrate organs are induced by FGF signaling between mesenchymal and epithelial cells across the layer of basal lamina that separates them. *Drosophila* basal lamina is similar in appearance and composition to vertebrate basal lamina [21] and is assumed to function in similar ways. *Drosophila* tracheal tubes are composed of a unilayered cellular epithelium whose basal (outer) surface has a layer of basal lamina. Imaginal discs have a similar organization. Tracheal responsiveness to ectopic Bnl-FGF reveals that Bnl-FGF can signal through the layers of basal lamina that surround both the imaginal disc and the trachea (Figure 3d). These measures of FGF signaling are qualitative only and do not assess relative efficiency of signaling, but they do reveal that

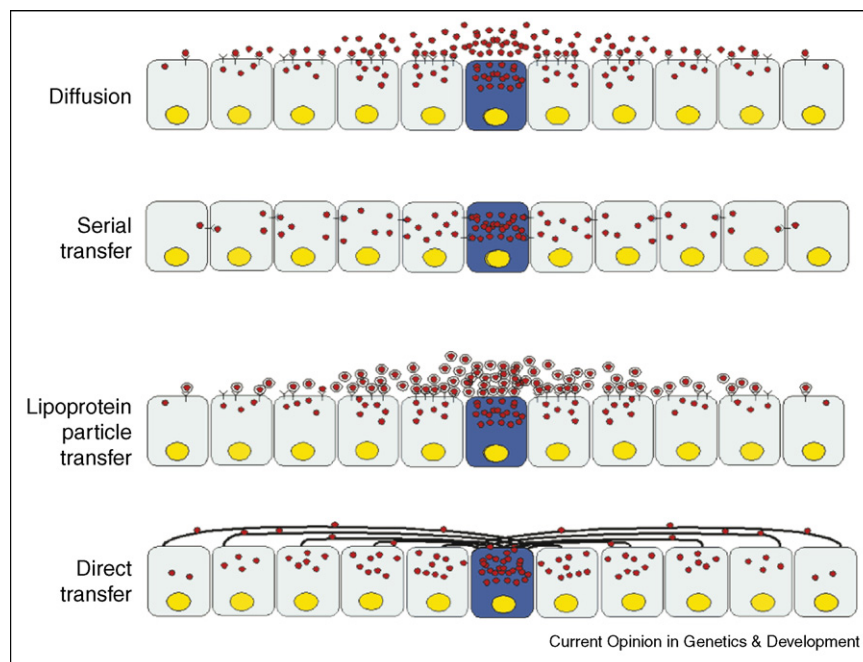
basal secretion of Bnl-FGF by wing discs is not confined by an impermeable barrier.

Movement of signaling proteins in the wing disc

The transparency of the *Drosophila* basal lamina to FGF signaling contrasts with the barrier functionality of the embryo plasma and vitelline membranes. The question arises whether transparency is a general property of basal lamina — whether the basal lamina is also transparent to Hh, Dpp, and Wg. No measures of Hh, Dpp, or Wg signaling through basal lamina have been reported, but if basal lamina were not transparent, it would presumably bind these proteins to restrict their movement. FGF binds its receptor together with heparan sulfate proteoglycans (HSPGs), one of the major constituents of extracellular matrix and basal lamina, so binding per se is not synonymous with barrier function. Wing disc associated tracheal cells express components of the Dpp and Wg signal transduction pathways and are sensitive to ectopic expression of these signaling proteins (L Lin, AG, TB Kornberg, unpublished); moreover, development of the disc associated trachea appears to be Hh dependent (L Lin, TB Kornberg, unpublished; [22]). It seems reasonable to assume, therefore, that tracheal cells have the capacity to respond to Hh, Dpp, and Wg if these proteins were to emanate from the disc (Figure 3c).

We propose that Hh, Dpp, and Wg move to form their respective gradients in a manner that prevents their

Figure 4



Four models of morphogen dispersion. Movement of morphogen (red) from source cell (middle, purple) to outlying cells by diffusion, serial transfer (transcytosis), lipoprotein particle transfer, and directly (via cytonemes).

contact with cells other than their intended targets. How might this be achieved? One possibility is that morphogen secretion is only apical, thereby preventing secreted morphogens from contacting potential targets such as the tracheal cells that are near the basal surface. Although the literature is not unanimous regarding the polarity of secretion, evidence for apical secretion in wing discs has been reported for Hh [17^{••},23,24], Dpp [18], and Wg [19,25^{••}].

If secretion is apical, how might cross-luminal signaling be prevented? Considering the apical dispersion of Dpp (Figure 3a and b), Dpp is expressed by the anteroposterior organizing signaling centers of the columnar and peripodial surfaces. If it is secreted in a form that diffuses freely, it will move until it is either degraded or bound by a receptor, co-receptor, or other type of binding protein. Unrestricted apical diffusion would presumably lead to activation of targets in both cell layers (Figure 3b). We assume therefore that dispersion is confined to the apical surface of both cell layers, such that it will activate targets only in the layer that produces it (Figure 1a).

Four types of mechanisms have been proposed to explain how morphogens move from source cells to targets in the wing primordium. As schematized in Figure 4, these are diffusion in extracellular space [26,27]; serial transfers from neighbor to neighbor involving transcytosis and endocytic trafficking [28,29^{••}]; transfer in lipoprotein particles [5,25^{••}]; and direct transfer at sites of cytoneme-mediated contacts [30,31]. No published experiments definitively establish any of these proposed mechanisms as operative (or inoperative), and it is beyond the scope of this brief essay to review how transcytosis, lipoprotein transfer, or direct contact might effect planar dispersion of morphogens. We posit that passive diffusion, whether it is entirely unfettered or involves shuttling between binding moieties in the apical membrane of the epithelial cells [2,23,32–37], is an unlikely mechanism to move morphogens for long distances in the plane of the epithelium if it cannot prevent them from moving even a short distance out of the plane.

Concluding remarks

There are two types of environments in which morphogen signaling gradients communicate information. One type is represented in the *Drosophila* oocyte and pre-cellular embryo. At these developmental stages, although the different signaling proteins are produced and function in a variety of ways, they each appear to disperse to form informational gradients by passive diffusion, either within the confines of the narrow space that surrounds the oocyte and embryo or within the syncytial cytoplasm of the embryo. The second type is probably typical of most cellular systems in which Hh, Dpp, and Wg function. It has closely juxtaposed cell populations that represent distinct developmental fields that are not insulated from

each other by impermeable barriers. We argue that the mechanisms that generate protein gradients in these systems must restrict signaling to the intended target fields despite the close proximity of other cells, conditions that are not likely to be compatible with passive diffusion.

Acknowledgements

We thank members of our lab as well as our colleagues Gail Martin, Didier Stainier, Jeremy Reiter, and Chester Chamberlain for helpful comments on the manuscript, and we acknowledge the National Science Foundation and the National Institutes of Health for funding.

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