DEVELOPMENTAL BIOLOGY

Direct Delivery Mechanisms of Morphogen Dispersion

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This Presentation focuses on how morphogen signaling proteins disperse across developmental fields. Although the steady-state distributions of morphogen signaling proteins have been described well in a number of contexts, the mechanisms that generate these distributions have remained uncertain. Results presented here show that these proteins transfer from producing to target cells at points of direct contact, even when the producing and target cells are not immediate neighbors.

Presentation Notes

Slide 1: Science Signaling *logo*

The slideshow and notes for this presentation are provided by *Science Signaling* (http://www.sciencesignaling.org).

Slide 2: Direct delivery mechanisms of morphogen dispersion

The idea of morphogen gradients dates to the origins of experimental embryology. In 1952, Alan Turing published a theoretical treatment of morphogen gradients that was highly influential (*1*).

Slide 3: The Turing hypothesis

Alan Turing was a British mathematician who is widely considered to be the father of computer science. His interest in mathematical biology led to a proposal for the chemical basis of morphogenesis and a model with two key aspects—the existence of chemical morphogens that elicit concentration-dependent responses and a mechanism for distributing morphogens in a concentration gradient (*1*). Progress in experimental biology has now succeeded in identifying chemical morphogens—proteins such as Bicoid, Decapentaplegic (Dpp), Hedgehog (Hh), and Wingless (Wg)—that have the properties Turing predicted. However, the proposal that diffusion is the basis for their distribution has not been verified experimentally, and we suggest an alternative model.

Slide 4: Morphogen gradients

Lewis Wolpert has made seminal conceptual contributions to our understanding of morphogens, having proposed the "French flag" model to illustrate that signaling in the form of a morphogen concentration gradient must elicit discrete responses across its target field of cells (*2*). This figure depicts a concentration gradient over a developmental field (shades of green) and specific transcriptional responses by cells (indicated by the red, orange, and blue nuclei) to different concentration ranges of the morphogen (*3*).

Slide 5: The A/P axis of the Drosophila wing imaginal disc is directed by a developmental organizer embodied by Dpp (BMP) protein

Groups of source cells that produce morphogens function as classical "developmental organizers"—they regulate the growth and regional specification of their client developmental field by distributing morphogen to target cells. The cells in the *Drosophila melanogaster* wing disc that produce the bone morphogenetic protein (BMP) Dpp at the anterior/posterior (A/P) compartment boundary function as a classic developmental organizer. These cells secrete Dpp, which becomes distributed in a concentration gradient that declines with increasing distance from the source cells (*4*).

Slide 6: Dpp targets in the wing disc

Targets of Dpp signal transduction in the wing imaginal disc—*spalt* and *omb* are shown here—are expressed in response to Dpp that emanates from the Dpp-producing cells at the A/P compartment boundary. *omb* is expressed more broadly than *spalt*, presumably because *omb* transcription is activated by a lower amount of Dpp signal transduction relative to that which induces *spalt* transcription (*5*).

Slide 7: What is the mechanism that disperses morphogens across a developmental field?

We argue that a mechanism of gradient formation based on passive diffusion is incompatible with the sensitivity requirements and geometries of the biological systems that use morphogen gradients to regulate their growth and patterning.

Slide 8: Evidence for direct signaling in the wing imaginal disc, eye imaginal disc, and trachea

We have studied the movement of morphogen signaling proteins in three organs of the third instar *Drosophila* larva—the wing imaginal disc, the eye imaginal disc, and the branches of the second tracheal metamere, which are associated with the wing disc. In each of these organs, we have correlated signaling with cellular extensions we call "cytonemes"—specialized signaling filopodia that appear to link target cells to sources of signaling proteins.

Subsequent slides will feature images of each of these tissues, and the diagrams on this slide illustrate relevant patterning events and landmarks in each tissue. As mentioned previously, a key feature of the wing disc is its band of Dpp-secreting cells along the A/P compartment boundary. A prominent feature of the eye disc is the morphogenetic furrow, a transient indentation in this columnar epithelium that moves across the disc in a posterior-to-anterior wave, leaving organized ommatidia in its wake. Each ommatidial group of cells has a polarity to its organization, and the "equator" refers to the imaginary line that is oriented perpendicular to the morphogenetic furrow and across which ommatidia on either side are oriented with mirror-image symmetry. The larval tracheal system serves as a blueprint for and contains the precursors of the adult tracheal system. The air sac primordium (ASP) is a tube that will produce the dorsal air sacs of the adult. It forms during the third instar period and is induced by Branchless (Bnl), which is a fibroblast growth factor (FGF) that is produced by a small group of cells in the wing disc that are shown in blue. The ASP is the only example in *Drosophila* of tubulogenesis that involves both cell proliferation and morphogenesis. All other tubes arise postmitotically.

Slide 9: Cytonemes in the wing disc

Cytonemes were discovered during the course of an enhancer trap screen that was carried out by Felipe-Andrés Ramírez-Weber, a postdoctoral fellow in the lab (*6*).

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The screen involved crossing flies carrying random insertions of a *Gal4*-containing transposable P element to flies carrying a reporter gene encoding green fluorescent protein (GFP) under the control of a Gal4 upstream activating sequence (UAS), and examining patterns of GFP expression in wing discs. One of the lines exhibited the interesting pattern shown in the left panel: Only cells along the flanks of the disc had GFP, suggestive of regulation by Dpp. Subsequent work has shown that the P element in this enhancer trap line was inserted in the (then unknown) gene *brinker* and that *brinker* expression is negatively regulated by Dpp. The feature that caught our attention was the presence of GFP in strands emanating from the GFP-expressing cells and extending toward the Dpp-secreting organizer cells of the A/P boundary. The term "cytoneme" refers to the fact that these extensions contain cytoplasm ("cyto") and are finger-like ("neme").

Slide 10: Cytonemes extend from the apical surfaces of wing disc cells and orient toward the A/P organizer

Cytonemes lie along the apical surface of the wing disc columnar epithelium. They can be seen emanating from clones of cells that express a membrane-tethered GFP, and they extend toward the Dpp organizer from either side of the Dpp organizer (*7*).

Slide 11: Dependence of A/P wing disc cytonemes on Dpp signaling

The orientation of apical wing disc cytonemes toward the Dpp organizer is dependent upon the presence of Dpp. In wing discs from flies that carry temperature-sensitive alleles of *dpp* (dpp^{ts}) and express CD8-GFP (a fusion of GFP and the transmembrane domain of CD8) in the *brinker* domain, cytonemes orient normally toward the Dpp organizer at the permissive temperature, but not at the nonpermissive temperature (*8*).

Slide 12: Apical cytonemes in the wing disc change shape in response to ubiquitous Dpp

Ubiquitous Dpp causes a change in the number, appearance, and orientation of wing disc cytonemes (*8*). After ectopic expression of a heat shock–inducible transgene encoding Dpp, the long, oriented cytonemes that normally emanate from CD8-GFP–expressing clones (left panel) are not present. Instead, many short cytonemes extend outward without apparent directional bias (right panel).

Slide 13: Apical wing disc cytonemes orient toward discrete ectopic Dpp sources, revealing the plasticity of cytoneme orientation

To characterize the relationship between apical wing disc cytonemes and cells that produce Dpp, Sougata Roy developed a two-clone system that generates two types of somatic clones—one that depends on flippase-catalyzed recombination and another that depends on Cre-catalyzed recombination (*7*). By titrating the activities of these recombinases, discs can be obtained that have a limited number of clones of each type. In wing discs with independent clones expressing either CD8-GFP or Dpp-Cherry, cytonemes emanating from GFP-positive clones that are close to Dpp-Cherry–expressing clones do not orient toward the disc's Dpp organizer, but instead orient toward the cells that ectopically express Dpp-Cherry.

Slide 14: The distribution of the Dpp receptor Tkv (Thickveins) in the wing disc is not restricted to the cell bodies

Expression of a GFP-tagged version of the Dpp receptor Thickveins (Tkv-GFP) in clones in the wing disc produces fluorescence in the cell bodies of the expressing cells and in puncta in outlying regions between the cell bodies and the Dpp organizer (*8*).

Slide 15: Conclusions I

From these experiments, we have shown that cells of the wing disc epithelium have cellular projections called cytonemes that emanate from the apical surface and orient toward the organizer. The presence and orientation of these cytonemes depends upon the production of Dpp by the organizer.

Slide 16: Eye imaginal disc

Regulation of growth and patterning of the eye disc is dependent upon several signaling proteins, including Dpp, Hh, Wg, and epidermal growth factor (EGF). The EGF Spitz is produced by cells in the morphogenetic furrow (*9*).

Slide 17: Eye disc cytonemes orient to morphogenetic furrow and to equator and respond to ectopic EGF *expression*

In CD8-GFP–expressing eye disc clones anterior to the morphogenetic furrow, cytonemes can be seen extending toward the morphogenetic furrow and toward the equator. No cytonemes have been observed in clones posterior to the furrow (*7*).

Ubiquitous expression of Spitz causes a change in the number and orientation of eye disc cytonemes. After ectopic expression of a heat shock–inducible transgene encoding Spitz, the long, oriented cytonemes that normally emanate from CD8-GFP–positive cells are not present. Instead, many short cytonemes extend outward without apparent directional bias (*7*), similar to what we observed with ubiquitous expression of Dpp in the wing disc.

Slide 18: Eye disc cytonemes contain EGFR

Expression of a GFP-tagged version of the EGF receptor (EGFR-GFP) in clones in the eye disc produces fluorescence in the cell bodies and in puncta that are present in cytonemes oriented toward the morphogenetic furrow. EGFR-GFP was not observed in cytonemes oriented toward the equator (*7*).

Slide 19: Conclusions II

There are two distinct types of cytonemes produced by cells of the wing disc epithelium: One orients toward the morphogenetic furrow, and the other orients toward the disc equator. Proper orientation of morphogenetic furrow–directed cytonemes depends upon production of the EGF Spitz being limited to cells of the morphogenetic furrow, and only cytonemes that orient toward the morphogenetic furrow contain the EGFR.

Slide 20: Wing disc and trachea make specific contacts

Several tracheal branches are associated with the third instar wing disc. The transverse connective branch of the second tracheal metamere (Tr2) extends underneath the disc basal lamina to deliver oxygen into the tissue.

Slide 21: Bnl (FGF) *expression in the wing disc*

During the third instar, the FGF Branchless (Bnl, the left panel shows in situ hybridization to *bnl* mRNA) induces outgrowth of the ASP branch, which will give rise to the dorsal air sacs of the adult fly (*10*). The dorsal air sacs are the largest tracheal organs of the adult and provide oxygen to the thoracic flight muscles.

Slide 22: ASP growth and morphogenesis require Bnl (FGF) signaling

Like other FGFs, Bnl induces signaling through the extracellular signal–regulated kinase (ERK). Staining with an antibody that recognizes the phosphorylated form of ERK (anti-dpERK) reveals active FGF signal transduction in the cells at the tip of the ASP (left panel). Expression of a dominant negative form of the FGF receptor (FGFR) Breathless (Btl^{DN}) stunts growth of the ASP (right panel) (*10*).

Slide 23: Cytonemes extend from the tip of the ASP to the disc cells expressing Bnl (FGF)

Expression of CD8-GFP in tracheal cells with a *btl-Gal4* transgene to drive expres-

sion of a *UAS:CD8-GFP* reporter labels the ASP and the cytonemes that emanate from its tip. These cytonemes orient toward the source of Bnl in the wing disc (*7*).

Slide 24: FGFR (Breathless) is in ASP cytonemes

Expression of a Btl-GFP fusion protein in tracheal cells also labels the ASP tip cytonemes and reveals that cytoneme-associated Btl is present diffusely throughout the shaft of the cytoneme and in discrete puncta along its length (*10*).

Slide 25: ASP cytonemes respond to ubiquitous Bnl (FGF)

Ubiquitous expression of Bnl causes a change in the number and orientation of ASP cytonemes. After ectopic expression of Bnl under the control of a heat shock–inducible promoter, the long, oriented cytonemes that normally emanate from the ASP tip are not present. Instead, many short cytonemes extend outward from the ASP in all directions (*10*).

Slide 26: ASP cytonemes orient toward clones ectopically expressing Bnl (FGF)

The ASP cytonemes were also characterized after ectopic expression of Bnl in clones of wing disc cells. In these two images, clones of Bnl-expressing cells are the dark areas that are visible against the background of red fluorescent protein (RFP)–expressing cells. The tracheal cells here express GFP and appear to respond to ectopic Bnl by extending cytonemes toward the clones (*10*). The two focal planes imaged in these panels show bright cytoneme tips that appear to touch the Bnl-expressing cells.

Slide 27: Conclusions III

The expression of Bnl (FGF), the expression of FGFR, and the presence and orientation of ASP cytonemes correlate precisely with FGF signaling.

Slide 28: Ligand specificity of cytonemes

The observed ligand-specific responsiveness of cytonemes leads to questions about their distinctiveness.

Slide 29: Cytoneme specificity

Previous slides showed how heat shock–induced expression of Dpp, Spitz, and Bnl caused dramatic changes in the size and orientation of cytonemes in the wing disc, eye disc, and ASP, respectively. The images on this slide document cytonemes on the apical surface of the wing and eye discs and at the ASP tip after heat shock–induced expression of Dpp (HS-Dpp), Spitz (HS-Spi), Bnl (HS-BNL), or Hh (HS-Hh). Notable changes to the cytonemes were only observed for the conditions represented by the panels that are highlighted by white outlines (*7*). These results show a pronounced specificity of cytonemes for particular signaling proteins—wing disc cytonemes for Dpp, eye disc cytonemes for Spitz, and ASP cytonemes for Bnl.

Slide 30: Ligand specificity

The unexpected discovery of cytoneme ligand specificity leads to the question of its mechanistic basis.

Slide 31: Signaling from disc to trachea

Our evidence implicates both Dpp and Bnl (FGF) as essential regulators of the ASP, leading us to ask whether cytonemes in this context can participate in only one or in both of these signaling pathways.

Slide 32: Tracheal cells are adjacent to Dpp*-expressing disc cells*

The image in the panel on the right shows air-filled tracheal tubes (red) and wing disc cells that express Dpp (green). The ASP is not yet filled with air at this stage, but we have outlined its periphery in blue. The ASP is closely juxtaposed to Dpp-expressing disc cells, suggesting that the ASP might be responding to Dpp produced in the disc.

Slide 33: Dpp is required for ASP morphogenesis

An antibody directed against the Discs large (Dlg) protein highlights basal-lateral membranes in red. Air-filled trachea are in green, and the periphery of each ASP has been outlined in purple. The normal ASP in the left panel extends outward from the transverse connective with a slender proximal stalk and club-shaped distal end. In a dpp^{ts} genetic background, ASP morphology is abnormal, showing bifurcations such as those shown in the right panels. Dpp is not produced by the ASP; the nearest cells that could be the source are in the wing disc.

Slide 34: Receptor localization in cytoneme subtypes

The top panel shows that expression of *UAS:Tkv-GFP* under control of the *btl-Gal4* driver illuminates ASP tip cytonemes both along the cytoneme shafts and in puncta. The middle panel shows that expression of *UAS:Btl-Cherry* under control of the *btl-Gal4* driver also illuminates ASP tip cytonemes both along the shafts and in puncta. The bottom panel shows that Tkv-GFP and Btl-Cherry localize to different ASP tip cytonemes (*7*). Although the mechanism that segregates the Dpp and Bnl receptors into different cytonemes is not known, the existence of cytoneme subtypes suggests that receptor localization is the basis for cytoneme specificity.

Slide 35: Distributions of Tkv-GFP and FGFR (Btl)-Cherry in cell bodies of ASP

This lower-magnification and lower-resolution image of the distal ASP does not resolve cytonemes, but puncta that contain either Tkv-GFP (green) or Btl-Cherry (red) are visible. Each of the large cells has several fluorescent puncta; the absence of overlap of fluorescence indicates that the Dpp and Bnl receptors localize separately in the cell bodies as well as the cytonemes (*7*).

Slide 36: Properties of cytonemes

The properties of cytonemes in the wing disc, eye disc, and ASP are consistent with roles in signaling and in transporting signaling proteins from source to target cells.

Slide 37: Tracheal cytonemes take up Dpp

The orientation, plasticity, and specificity of cytonemes imply that cytonemes could traffic signaling proteins from producing cells to target cells. Showing this function directly in the ASP context was accomplished by expressing Dpp-GFP in the wing disc with *dpp-LexA*::*lexO*:*dpp-GFP* and expressing Tkv-Cherry in the ASP with *btl-Gal4*::*UAS:Tkv-Cherry*. The swath of green fluorescence in the image on the right is emitted by disc cells that are out of the focal plane. Puncta in the tracheal extensions show both red and green fluorescence, indicating uptake of Dpp from the disc cells. Most of the visible cytonemes have both red and green fluorescence, indicating that Tkv-containing cytonemes take up Dpp and suggesting that cytonemes that lack Tkv are unable to take up Dpp.

Slide 38: Functional differences between ASP cytonemes

This is a higher-magnification view showing uptake of Dpp-GFP from disc cells into CD8-Cherry–containing cytonemes. All cytonemes are marked with CD8-Cherry, but only some contain Dpp-GFP.

Slide 39: Cytonemes summary

The remarkable properties of cytonemes reveal that they adopt different forms and functions in various different signaling contexts.

Slide 40: Cytonemes and Hedgehog signaling

Recent work from the lab of Isabel Guerrero shows that in posterior compartment cells of the wing disc, Hh is packaged into vesicles that exit from the basal-lateral surface of these epithelial cells and move outward along basal cytonemes (*11*). This work complements our studies of cytonemes on the apical surface of disc cells that are responsive to and appear to traffic Dpp. Together, these findings suggest that the

polarized columnar epithelial cells of the wing disc have apical extensions that take up Dpp and basal extensions that deliver Hh. That is, these cells have distinct asymmetric extensions that specifically receive or deliver signaling proteins.

Slide 41: The basis for cytonemes

The signaling characteristics of cytonemes indicate that they are endowed with many singular capacities.

Slide 42: Precedents from neurobiology

We can recognize the distinctive capacities of cytonemes in established features of neurons, and suggest that cytonemes may be functionally equivalent to axons and dendrites.

Slide 43: Conclusions IV

The properties of cytonemes are consistent with a general role in the dispersion of signaling proteins.

Slide 44: Acknowledgments

This work represents the combined efforts over more than 10 years of these four postdoctoral fellows. We dedicate our efforts to the late Felipe-Andrés Ramírez-Weber (1964–2007).

Editor's Note: This contribution is not intended to be equivalent to an original research paper. Note, in particular, that the text and associated slides have not been peer-reviewed.

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