

Cytonemes: Cellular Processes that Project to the Principal Signaling Center in *Drosophila* Imaginal Discs

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Summary

Wing imaginal disc cells in *Drosophila* develop by using information received from a signaling center associated with the anterior/posterior compartment border. We show here that disc cells have thin, actin-based extensions (cytonemes) that project to this signaling center. Cytonemes can be induced when cells from the lateral flanks of a wing disc are cultured next to cells from the A/P border or next to a source of fibroblast growth factor. Mouse limb bud cells also grow projections during a brief culture period, indicating that cytonemes are an attribute of both vertebrate and invertebrate cells. We suggest that cytonemes may be responsible for some forms of long-range cell-cell communication.

Introduction

Cells use a variety of mechanisms to communicate over long distances. Information can be transmitted to distant organs by small proteins or organic molecules that travel to specific receptors at target sites. Alternatively, information can be transmitted by long cellular extensions such as axons. These neuronal processes transduce signals between the nerve terminal and cell body by actively transporting endocytosed ligands or by conducting electrical currents. Long distance communication also is involved in the development of epithelial cells, since their growth is controlled by signaling centers that can be located more than 100 μm away. However, the mechanisms that link these cells to the signaling centers are not understood.

In the *Drosophila* wing imaginal disc, the principal signaling center is located on the anterior side of the A/P compartment border that bisects the disc. It is created by signals emanating from the A/P compartment border and is defined operationally by its roles both in regulating growth and patterns of gene expression and in prescribing the placement of each pattern element (Basler and Struhl, 1994; Tabata and Kornberg, 1994). Two proteins, Hedgehog (Hh) and Decapentaplegic (Dpp), carry out the respective signaling functions of the compartment border and the signaling center it generates.

Hh is one of a small group of genes whose domain of expression is delimited by A/P compartment borders. It is expressed by all P compartment cells (Tabata et al., 1992) and produces a secreted protein (Porter et al.,

1995) that signals A cells (Lee et al., 1992; Tabata and Kornberg, 1994; Porter et al., 1995; Zecca et al., 1995; Mullor et al., 1997). A cells respond in part by expressing the *Dpp* gene in a narrow stripe of cells on the anterior side of the A/P border (Figure 1A; Basler and Struhl, 1994; Tabata and Kornberg, 1994). The Dpp protein is also secreted. Dpp is believed to embody the activity of the A/P signaling center (Capdevila and Guerrero, 1994; Zecca et al., 1995) by using a concentration-dependent mechanism to control the expression of various target genes (Lecuit et al., 1996; Nellen et al., 1996; Tsuneizumi et al., 1997). Although Dpp is assumed to form a concentration gradient, there is no direct evidence for its distribution in discs. Nevertheless, its target genes are expressed in roughly symmetric patterns with respect to the A/P border, and as a consequence, A and P cells in the fly wing differentiate particular structures at prescribed distances from the border. Since cells at equivalent distances on either side of the compartment border have the capacity to make identical structures (Garcia-Bellido and Santamaria, 1972; Lawrence and Morata, 1976), they are said to behave as though polarized with opposite orientations. This use of the term polarity is unrelated to apical/basal or dendritic/axonal polarity, or to the “planar polarity” that orients the hair and bristle structures that epidermal cells make. It has had no known morphological manifestation.

In addition to defining where key regulatory genes are expressed and generating an associated signaling center, the A/P compartment borders also confine imaginal disc cells to either side (Garcia-Bellido et al., 1973, 1976; Morata and Lawrence, 1975, 1978; Steiner, 1976; Lawrence et al., 1979; Kornberg, 1981; Struhl, 1981) and can retard the diffusion of small organic molecules (Weir and Lo, 1982). This partial catalog of the functions associated with the compartment border illustrates some of the ways in which cells at the compartment border generate spatial guideposts during development. It seems reasonable to predict that these cells may have special structures to perform these tasks, but no distinguishing morphological structures that might endow them with their special functions have been reported.

The study described here was guided by our interest in learning more about the nature of the border cells. In particular, we have been examining how the Hh and Dpp proteins signal target cells. Although the influence of Dpp extends to the edges of the wing disc (Capdevila and Guerrero, 1994; Zecca et al., 1995) and Hh can signal across long distances as well (Chen and Struhl, 1996), these proteins share an unexpected characteristic: they do not move efficiently in the extracellular environment. Current evidence indicates that the active form of Hh has cholesterol covalently bound at its C terminus (Porter et al., 1996) and an N terminus that is palmitoylated (Pepinsky et al., 1998). Both modifications are likely to anchor Hh in the membrane of the cell in which it is made. The Dpp homolog, TGF β , binds to extracellular matrix proteins (Taipale and Keski-Oja, 1997) and is not

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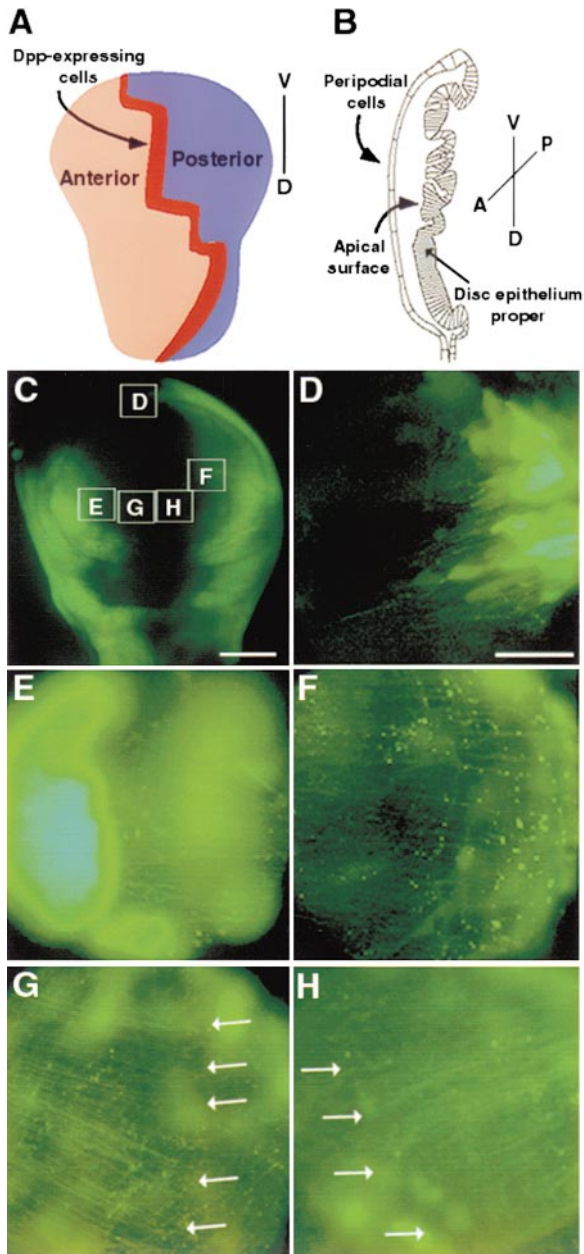


Figure 1. Cells of the Third Instar Wing Imaginal Disc Have Long Polarized Cytonemes

Drawings of wing discs in frontal view (A) and cross section (B) illustrate how the folded epithelium packs columnar cells to one side and arranges Dpp-expressing cells at the juxtaposition of the A and P compartments. (D–H) Fluorescent micrographs of five discs obtained from the *3S-GAL4;UAS-GFP* strain. Although cells in the disc epithelium proper that express GFP are limited to the lateral flanks, thin threads of fluorescence can be seen in the central regions adjacent to GFP-containing cells. The lettered boxes in (C) refer to the approximate locations represented by the higher magnification views in (D)–(H). Arrows in (G) and (H) mark the locations where the cytonemes appear to terminate. Scale bars represent 50 μm in (C) and 10 μm in (D). Panels (D)–(H) are at the same magnification. All discs in this and subsequent figures are oriented anterior left and dorsal down.

expected to diffuse readily from the cells in which it is synthesized. Simple diffusion thus appears inadequate to distribute these proteins over long distances or even short distances. Alternative mechanisms that have been considered include serial passage through neighboring cells (Strigini and Cohen, 1997; Bellaïche et al., 1998) and induction in target cells of still other signaling molecules that might move more freely in the extracellular environment. Here, we report that disc cells have a novel type of structure that may provide another means by which signaling molecules can be transported across fields of disc cells.

Results

Drosophila Cells with Long Cytoplasmic Extensions

In the course of an ongoing study to identify and characterize genes whose expression correlates with compartments and compartment borders, we screened fly strains that harbor enhancer trap transposons at various locations in their genome. Using a green fluorescent protein (GFP) reporter to illuminate cells in which an enhancer trap transposon is transcriptionally active, we identified a strain (*3S-GAL4*) that produces GFP in wing imaginal discs in the pattern shown in Figure 1C. Bright green fluorescence was present in two broad swaths along the anterior and posterior flanks of the disc epithelium. No GFP-containing cells were present in the region where the A/P compartment border is located. Careful examination of these discs at high magnification revealed, however, that green fluorescence was not confined to the lateral flanks.

Wing discs were dissected from late third instar larvae, mounted without fixatives, and flattened under the slight pressure of a standard coverslip in contact with a microscope objective. In these preparations, faint strands of fluorescence could be seen in the central region of the disc (Figures 1D–1H). These thin fluorescent projections appeared to emanate from the GFP-containing cells along the lateral flanks and were oriented toward the disc center. Similar projections were also observed in third instar leg imaginal discs and in wing discs obtained from late second instar larvae (data not shown). These projections were consistent in appearance and were remarkably long and thin, yet their fluorescence was generated by a form of GFP that was cytoplasmic and was not coupled to another protein. Since they represent extensions of cell cytoplasm, and to indicate their thread-like nature (neme = thread), we designate them cytonemes. Numerous processes called variously filopodia, thin filopodia, and lamellapodia have been described previously. These processes vary in diameter, length, and kinetics of growth. We designate the *Drosophila* processes cytonemes to distinguish them as filopodia of a special type, ones with a diameter of approximately 0.2 μm (see below) and that are polarized with respect to their orientation to an organizing influence.

A wing disc is composed of an epithelial sheet that invaginates from the embryo epithelium to form a single-layered sac; its luminal (apical) surface becomes exposed to the exterior after metamorphosis. In the late third instar disc, severe flattening creates two apparent

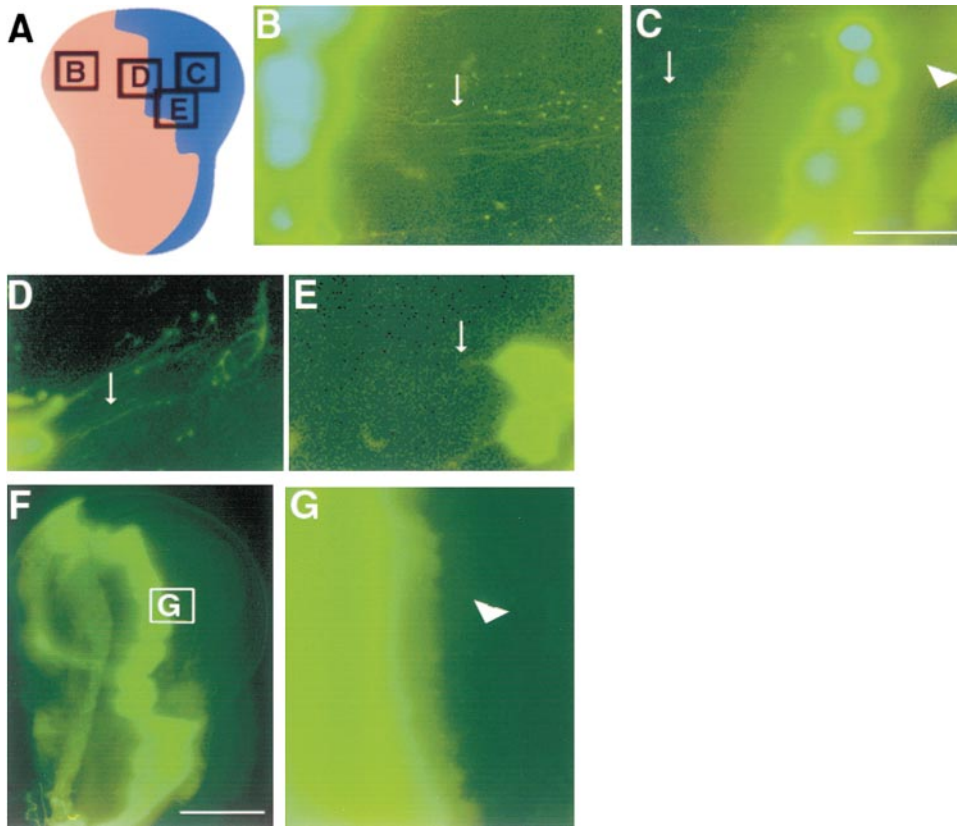


Figure 2. Cytonemes of Wing Disc Cells Orient toward the A/P Compartment Border

GFP-expressing cells in somatic clones at various random locations in a third instar wing disc (boxes in [A] and [B]–[E]) have cytonemes oriented toward the disc center (arrows) but not away from the disc center. Expression of GFP driven by the *ptc-GAL4* line is primarily at the A/P compartment border of third instar discs (F); no cytonemes could be seen emanating from these cells (G, arrowhead). Scale bars in (B), (C), (E), and (G), 10 μm ; scale bar in (D) and (F), 50 μm .

layers and minimizes the luminal cavity (Figure 1B). The “peripodial” side is sparsely populated with thin, squamous cells. In contrast, the “disc epithelium proper” on the other side is densely packed with cells that are highly columnar (0.5–2 $\mu\text{m} \times 20\text{--}30 \mu\text{m}$; Ursprung, 1972). The disc measures approximately 300 $\mu\text{m} \times 450 \mu\text{m}$, and several folds of the disc epithelium proper help to accommodate its estimated 60,000 cells. Second instar discs measure approximately 125 $\mu\text{m} \times 180 \mu\text{m}$; their cells are cuboidal, and although fewer in number, have apical surfaces that are significantly larger than the cells of third instar discs. In both second and third instar discs of *3S-GAL4* larvae, cytonemes are visible in focal planes just above the luminal, apical surface of the cells in the disc epithelium proper.

Cytonemes could not be observed after addition of a fixative such as formaldehyde, or after any lateral movement of the preparation once the microscope objective made contact with the coverslip. We were therefore not able to scan across an entire third instar wing disc but were limited to examining a small portion. Figures 1C–1H show a composite of high magnification views from five locations of five different discs and superimpose them on a single lower magnification photo. This montage demonstrates that cytonemes are long, polarized, and remarkably straight. Due to their great lengths, cytonemes from the GFP-containing cells on the disc flanks

extend beyond the field of view and could not be followed to their ends. Views of the middle region of the disc suggest that cytonemes emanating from the lateral regions terminate in the area of the A/P compartment border (Figures 1G and 1H, all arrows), although we were not able to mark the border independently. In this central region, fluorescent threads in various oblique orientations could be seen; these threads may reflect arborization of the cytonemes (Figures 1G and 1H). The cytonemes emanating from the disc flanks seemed not to meet at a distinct point. Rather, they left a region in the center of the disc devoid of processes oriented perpendicular to the A/P border (Figure 1G). Similar preparations of late second instar wing discs suffered from higher background fluorescence, but the smaller size and flatter epithelial surface of these discs made it possible to view both the lateral disc flank and central border region at once. The distribution and appearance of the cytonemes in these second instar discs were the same as in the third instar discs.

We detected cytonemes in two ways. They were observed either when cells along the lateral flanks contained GFP (Figures 1D–1H and 2B) or when small clones of GFP-containing cells were present at various random locations more than 50 μm from the A/P border of third instar discs (Figures 2C and 2E). GFP-containing cytonemes were observed that were oriented toward the

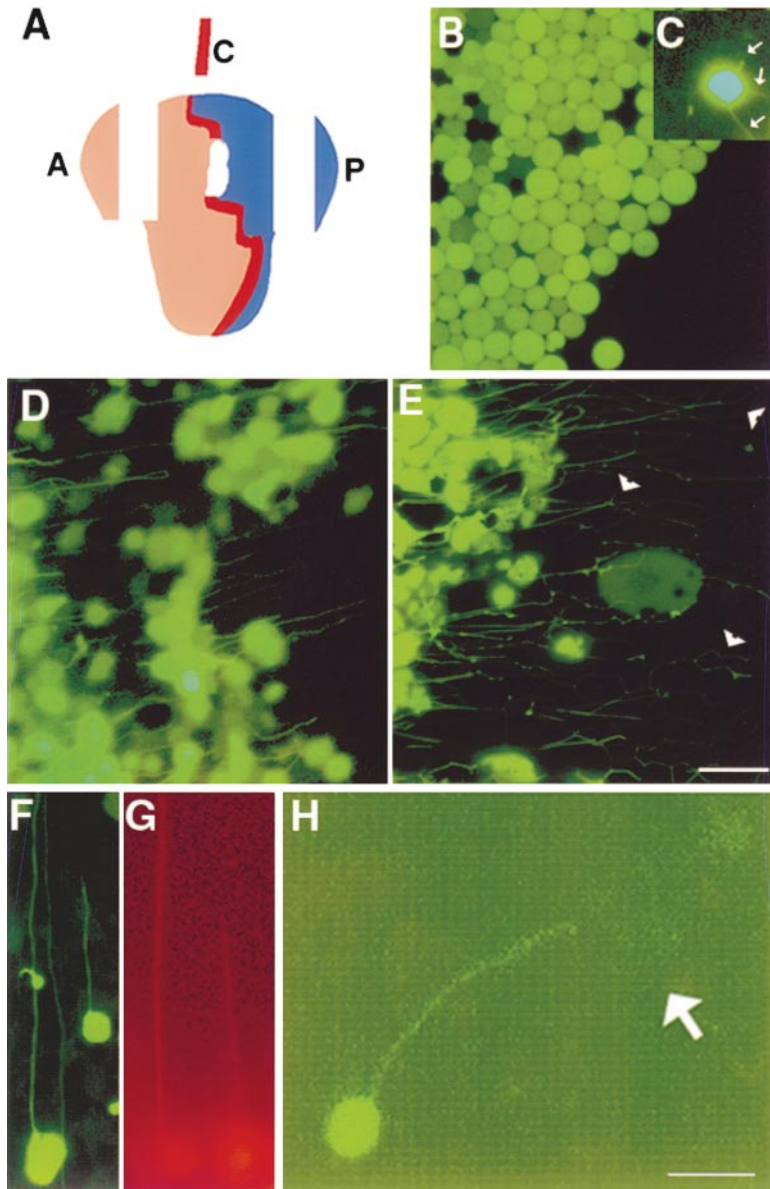


Figure 3. Cytosomes Grow during Culture

Fragments were cut from late third instar wing discs as shown in (A) and examined with fluorescence microscopy. When placed on a coverslip, GFP-containing cells were round (B), but after approximately 40 min of culture, multiple small processes containing GFP emerged (C). These processes extended and retracted rapidly. After approximately 60 min, the cells produced long processes containing GFP that oriented in one direction (D–H). The C fragment cells are not fluorescent and cannot be seen in these micrographs, but their locations are reflected in the orientation of the cytonemes that grow toward them and in the branching of the processes where the cytonemes make contact (E, arrowheads). (F) Cells from a more dispersed preparation showing a single long cytoneme per cell. (G) Cytonemes bind phalloidin-rhodamine. (H) Arrow points to a nonfluorescent S2 cell expressing dFGF that has attracted the growth of a cytoneme from a GFP-containing A fragment cell. Scale bar, 10 μ m.

disc center (Figure 2C, arrow), not away from it (Figure 2C, arrowhead). They were not visible when GFP was expressed by cells near the border, either in GFP-expressing clones (data not shown) or in cells expressing GFP under control of the *patched* (*ptc*) promoter (Figures 2F and 2G). We conclude that cytonemes extend from disc cells toward the A/P compartment border, but not from A/P border cells outward.

Inducing Cytonemes

The polarized distribution of cytonemes suggests that wing disc cells grow cytoplasmic extensions in response to a chemoattractant. To test this possibility and to further characterize the cytonemes, we developed a method to induce them in a culture of wing disc cells. Small pieces of third instar wing discs were isolated and monitored during a brief culture period of 50–90 min.

(Figure 3A). Fragments from the anterior flank of the wing pouch (A fragments) grew few or no cytonemes when cultured alone or next to another A fragment or a fragment from the posterior flank of the wing pouch (P fragment; Table 1). P fragments were similarly unresponsive, as were fragments from the central region of the wing pouch (C fragment) when cultured alone. Cytonemes proliferated abundantly, however, if an A or a P fragment was cultured next to a C fragment (Table 1). After a period of about 40 min during which few changes were apparent (Figure 3B), A or P cells produced multiple cytoplasmic extensions that were short, randomly oriented, and transient (Figure 3C). At approximately 60 min, the appearance of the cell culture changed dramatically, as long cytonemes grew from the A or P cells with a rapid burst (≥ 15 μ m/min, see Experimental Procedures). These extensions were long lived, and all were

Table 1. Cytoneme Growth by Cultures of *Drosophila* and Mouse Cells

Tissue ^a	Response ^b	Experiments (No.)	Experiments (% with Response) ^c
A + A	+	5	100
P + P	+	4	100
C + C	+	3	100
A + C	+++	32	75
P + C	+++	15	80
A + eye	–	3	100
A + C _{leg}	+	1	100
P + C _{leg}	+++	3	100
A _{leg} + C	+++	5	100
A _{ant} + C	+++	2	100
A + S2	–	8	100
A + S2 _{Dpp}	–	3	100
A + S2 _{Hh}	–	4	100
A + S2 _{dFGF}	+++	9	89
P + S2 _{dFGF}	+++	6	83
A + C _{hhs}	–	6	0
P + C _{hhs}	–	3	0
A _{hhs} + C	+++	2	100
P _{hhs} + C	+++	4	100
embryo _{wild type}	+++	15	80
embryo _{bt1}	+++	8	100
embryo _{bnf}	–	12	100
mouse limb bud	+++	5	100

^aUnless indicated otherwise, the tissue fragment was from a wing imaginal disc.

^bEstimates of number of cytonemes: (–) none, (+) very few, (+++) many.

^cIn experiments that did not give the indicated response, no cytonemes were observed.

oriented toward the C cells (Figures 3D and 3E). Their direction of growth was substantiated by direct observation and by their GFP content. Fluorescent cytonemes were present only if the A or P cells contained GFP, and were not present if just the C fragment cells contained GFP.

A and P cells formed single processes that were remarkably straight and could extend for distances many times the diameter of the disc cell (Figure 3F); some were 700 μ m. The processes appeared to contact C fragment cells and to arborize. These cytonemes could be detected with differential interference contrast optics as well as fluorescence optics (Figures 4B–4G), allowing us to estimate their maximum diameter at the limit of resolution, 0.2 μ m (see Experimental Procedures). They did not survive any method we used to add fixative but grew unhindered in the presence of nocodazole, a microtubule-destabilizing drug. They could, however, be labeled with phalloidin-rhodamine (Figure 3G). We conclude that these cytonemes contain actin, and not microtubules.

We also developed a method to induce cytoneme growth in crude mixtures of *Drosophila* embryo cells. In such preparations, three cytonemes were seen in which a vesicle was observed to translocate a considerable distance away from the cell body. One of these was recorded photographically (Figures 4E and 4G). This indicates that materials can move rapidly away from the

cell body within these processes; we do not know whether materials in cytonemes can also move toward the cell body.

The ability of C fragment cells to induce polarized growth of cytonemes from A and P fragments suggests that C fragments are a source of a chemoattractant. To characterize this putative chemoattractant, we first asked whether it is uniquely localized in C fragments of wing discs. It is not. Although we were not able to identify a piece of an eye imaginal disc that is proficient at inducing cytonemes, fragments cut from the central, A/P border-containing region of the antennal disc or from leg discs could induce cytonemes in the same manner as wing C fragment cells (Table 1). The reciprocal experiment showed that A and P fragments cut from leg discs grew cytonemes in the presence of a wing C fragment. Since eye discs do not have an A/P compartment border or associated signaling center but both antennal and leg discs do, these results suggest that wing disc cells respond to a chemoattractant that is produced by signaling centers associated with the A/P border.

As described in the Introduction, the special properties of the cells at the A/P border are a consequence of Hh signaling, so we asked whether the ability of C fragment cells to attract cytoneme growth is dependent upon Hh. It is. C fragments from a *hh^{ts}* strain that had been incubated at the nonpermissive temperature (see Experimental Procedures) were unable to induce cytonemes from A or P fragments cut from *3S-GAL4* wing discs. In contrast, A or P fragments isolated from the *hh^{ts}* strain produced cytonemes normally when juxtaposed to C fragments isolated from normal wing discs (Table 1). This experiment shows that the C fragment cells require Hh; it does not identify Hh as the chemoattractant.

To identify the chemoattractant, we first asked whether any of the proteins with known signaling functions could substitute for C fragment cells in our assay. We placed A or P fragments next to a clump of S2 cells and established that this line of *Drosophila* tissue culture cells does not induce the growth of cytonemes under these conditions. S2 cells transfected with cDNAs encoding Hh or Dpp were similarly inactive (Table 1). We then asked whether FGF might function to stimulate cytoneme growth, since FGF has been implicated in polarized growth of neurons (McFarlane et al., 1995) and tracheal cells (Lee et al., 1996; Sutherland et al., 1996), as well as in cell migrations (Lee et al., 1996; Sutherland et al., 1996; Burdine et al., 1998). We examined the activity of FGF in two ways. We placed A fragment cells next to a heparin-coated acrylic bead that had been soaked in a solution containing vertebrate FGF4 (Niswander et al., 1993). After a period of culture, the *Drosophila* disc cells grew cytonemes in the direction of the bead (data not shown). This heterologous interaction was not tested further; instead, we tested the inductive capacity of the *Drosophila* FGF Branchless (referred to hereafter as dFGF; Sutherland et al., 1996) by placing A or P fragments next to S2 cells that had been transfected with dFGF cDNA. S2 cells expressing dFGF induced cytonemes that were indistinguishable from those induced by wing C fragment cells (Table 1; Figure 3H). We conclude that in our culture system, FGF can substitute

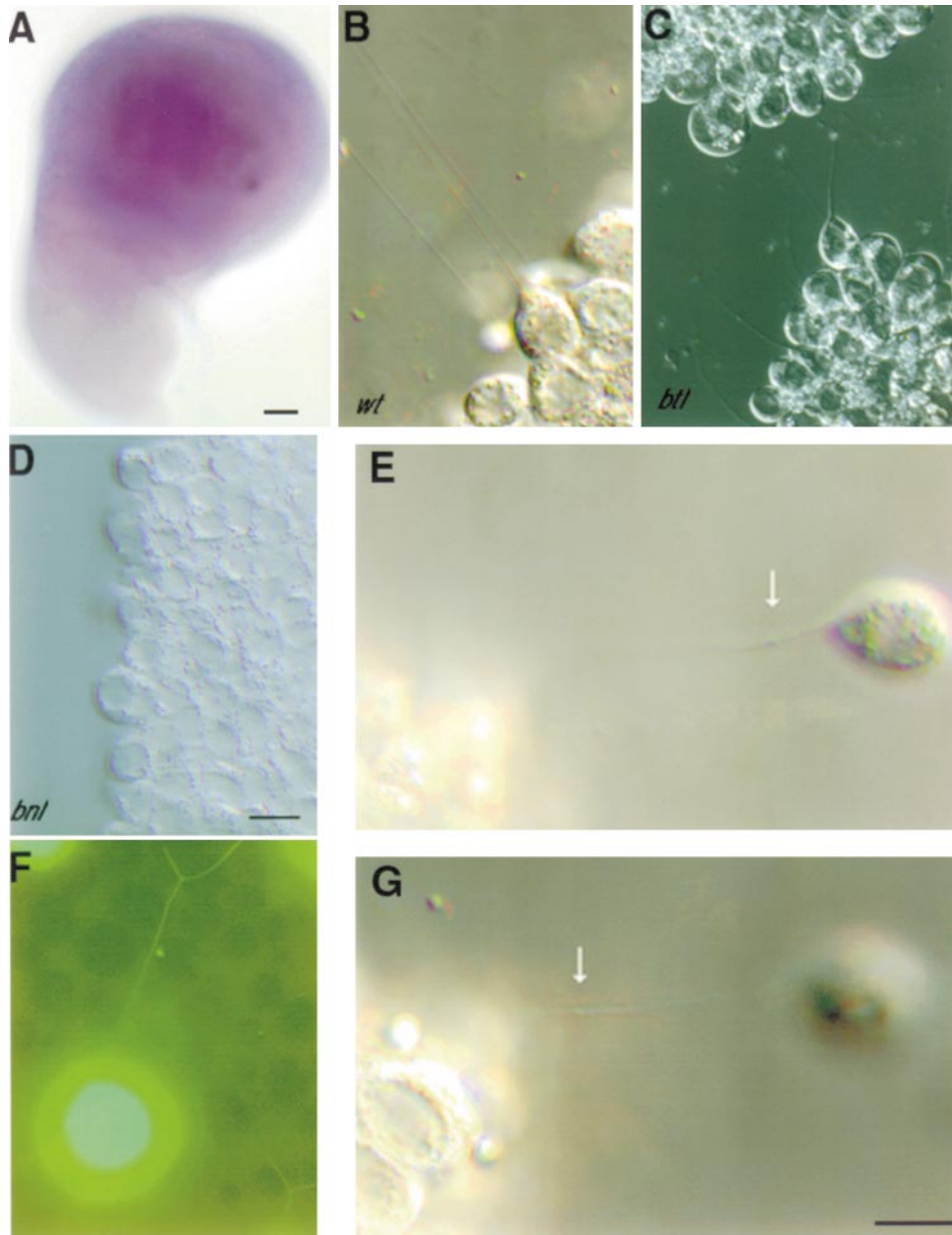


Figure 4. Cytonemes Grow Toward a Source of FGF

(A) In situ hybridization revealed that dFGF mRNA is relatively uniformly distributed in late third instar wing discs. Arrowheads indicate cytonemes that grew during culture of cells obtained from wild-type (B) or mutant embryos lacking the *breathless* dFGF receptor (C). Cells obtained from mutant embryos lacking dFGF (*branchless*) did not generate cytonemes of comparable size or abundance (D). Cytonemes can grow from mouse limb bud cells (F) and can transport vesicles (arrowheads in [E] and [G]). Scale bars are 25 μm in (A) and 10 μm in (B)–(G).

for C fragment cells to promote polarized growth of cytonemes.

Cytonemes and *Drosophila* FGF

Our studies of wing discs did not indicate whether cytonemes are short or long lived, but the experiments with the isolated disc fragments revealed that third instar imaginal disc cells retain the capacity to grow cytonemes in response to dFGF. This observation raises the possibility that dFGF is the agent that induces cytonemes in wing discs and that a gradient of dFGF orients

their growth. If so, cells near the A/P border might be a source. As shown in Figure 4A, in situ hybridization revealed that dFGF mRNA is present in third instar wing discs, but that its level is almost uniform. This pattern of dFGF mRNA expression does not suggest how a gradient of dFGF might form to induce cytonemes in discs.

In order to examine the role of dFGF further, we characterized cytoneme growth in a cell suspension prepared from wild-type and mutant embryos. Preparations from wild-type embryos produced cytonemes in random

orientations that were otherwise indistinguishable in appearance and growth kinetics from the disc cytonemes. However, when we prepared cell suspensions from embryos mutant for dFGF (*branchless*), the cytonemes we observed were unusually short and abnormally low in number (Figure 4D). In contrast, similar preparations from embryos mutant for the *breathless* dFGF receptor were indistinguishable from wild type (Figures 4B and 4C). Interpretation of these results is complicated by the presence of two genes in *Drosophila* that encode distinct FGF receptors (Beiman et al., 1996; Gisselbrecht et al., 1996) and by the fact that only a single gene encoding an FGF ligand has been identified (Sutherland et al., 1996). Without enumerating the ways by which multiple FGF receptors and ligands might affect growth of cytonemes, the ability of dFGF to induce them and the inability of mutants lacking dFGF to induce them in a normal manner are consistent with the proposal that dFGF is a chemoattractant that can induce and orient cytonemes.

We suggest two possible ways in which dFGF might function as a chemoattractant despite its not being expressed specifically by compartment border cells. dFGF protein has a large N-terminal region that is not characteristic of other members of the FGF family (Sutherland et al., 1996), and it is possible that dFGF must be post-translationally modified to generate active protein. If so, then active dFGF protein could be produced at the compartment border, and its distribution need not correlate with the presence of dFGF mRNA. Alternatively, it is possible that in the wing disc, dFGF functions primarily as a growth factor to stimulate nonpolarized growth of cytonemes. In this model, most cytonemes would have a short half-life and would grow randomly in a milieu in which the concentration of dFGF is uniform. However, if cytonemes contact cells that can provide an appropriate signal (e.g., Hh or Dpp), they could be stabilized and would make a functional junction.

dFGF is a member of a family of proteins that have critical roles in many developing systems. Examples include *Drosophila* tracheal cells, which extend toward a source of dFGF (Lee et al., 1996); sex myoblasts in *C. elegans*, which move toward sources of FGF (Burdine et al., 1998); and axons of *Xenopus* retinal ganglia, which depend upon FGF signaling for their directed growth (McFarlane et al., 1995). These observations raise the possibility that FGF may be eliciting cytonemes in these tissues in a manner analogous to its action with *Drosophila* imaginal disc cells.

Vertebrate Cytonemes

To ask whether cytonemes are present in other organisms, we made cell preparations from GFP-containing mouse limb buds. After an incubation under conditions that were identical to the cultures of imaginal disc fragments, we observed that filopodia grew with kinetics and appearance that were indistinguishable from *Drosophila* cytonemes (Figure 4F). Comparable results were also obtained with preparations of GFP-containing chick embryo cells (F.-A. R.-W. et al., unpublished results). These observations indicate that cytonemes are an attribute of both vertebrate and invertebrate cells and suggest that they may be a common attribute of cells in multicellular eukaryotes.

Discussion

Speculations on Cytoneme Function

The remarkable polarized orientation of cytonemes relative to the A/P compartment border in *Drosophila* imaginal discs suggests that cytonemes may help disc cells define their relationship to these signaling centers. Cytonemes that grow between cells in culture can achieve lengths that exceed an entire wing disc. They also orient their rapid growth toward, and appear to contact cells secreting, a chemoattractant. These features suggest to us that the cytonemes in discs link outlying cells to the disc centers, allowing cells of the A/P signaling center to directly contact more distant cells. These contacts could provide a mechanism for efficient, long distance transport.

Why have cytonemes not been seen previously? These structures are in fact very difficult to see. They are extremely fragile and are brought into focus only when discs are flattened in a precisely controlled manner. Having seen cytonemes in *Drosophila*, we now wonder whether similar extrusions exist in other organisms. Indeed, closely related structures have been observed in other systems that are optically more advantageous. Thin filopodia have been observed to extend 10–30 μm between epidermal cells in *Rhodnius* and *Calpodes* (Locke, 1987). Sea urchin embryos are relatively transparent, and filopodia and lamellapodia of various dimensions have been described (Gustafson and Wolpert, 1967; Karp and Solursh, 1985; Miller et al., 1995). Particularly noteworthy are the “thin filopodia” that connect primary mesenchyme cells with ectodermal cells. These structures were observed to grow as fast as 25 $\mu\text{m}/\text{min}$ to lengths as long as 80 μm ; they are remarkably straight and thin (0.2–0.4 μm in diameter) and contain actin. Although the filopodia do not exhibit the kind of polarization that characterizes the *Drosophila* cytonemes, there are clearly structural similarities.

The idea that body patterns are determined by morphogenetic gradients dates to the origins of experimental embryology, but convincing evidence for a molecular gradient that can generate scalar values which lead to different pattern elements along a body axis has come only recently. In the late 1980s, Nüsslein-Volhard and colleagues discovered that a monotonic gradient of Bicoid protein forms along the A/P axis of a *Drosophila* embryo (Driever and Nüsslein-Volhard, 1988); they and others showed that Bicoid acts as a morphogen in a concentration-dependent manner to determine where various body parts form (Driever et al., 1989). Bicoid is a transcription factor that is synthesized at the anterior pole of a newly fertilized embryo (Berleth et al., 1988); it is free to diffuse extensively because the embryo is a syncytium whose nuclei share a common cytoplasm. This method for generating a gradient of Bicoid protein does not immediately suggest a mechanism for distributing morphogens in the multicellular organs of subsequent developmental stages. Moreover, as noted above, the properties of the morphogens Hh and Dpp that organize wing imaginal discs prevent simple diffusion from distributing them to distant cells.

We propose that cytonemes may be responsible for distributing morphogens. Hh and Dpp are powerful morphogens that cause significant deviations from normal

anatomy when placed in ectopic locations. We hypothesize that their release from cells is at sites of cytoneme contact and is engineered both to deliver them efficiently and to limit their spread. If morphogen signals travel the length of cytonemes to generate a response in the target cell body, then the mechanisms that define distance from morphogen-producing cells need not require extracellular gradients. Instead, the morphogen, or a second message induced by the morphogen, may decay with time or distance as it is transported from the cytoneme tip to the cell body. The morphogen gradients in this model are intracellular.

In the foregoing descriptions and discussion, striking parallels are apparent between cytoneme-bearing imaginal disc cells and neurons. Both have long cytoplasmic extensions. In addition, both axons and cytonemes contact distant cells and arborize, and both respond to a chemoattractant that, at least in some cases, includes or is constituted by FGF (McFarlane et al., 1995). During development, ectodermal cells and neurons derive from a common pool of cells. Perhaps it should not be surprising that they share so many structural and functional aspects. The key point, however, is the possibility that cytonemes and axons might represent a general means by which cells communicate with their environment.

Experimental Procedures

In Situ Hybridization

A hybridization probe was prepared with a *bnl* cDNA clone and used for in situ hybridization according to the method described in Sutherland et al. (1996).

Microscopy

Wing imaginal discs were dissected from wandering third instar larvae with the genotype *3S-GAL4;UAS-GFP65T*. Discs were mounted on standard microscope slides, peripodial side up, and covered with an unsupported coverslip in PBS or S2 medium. As the disc compressed under the coverslip, a small percentage seemed to break and generate small membrane-bound vesicles containing GFP. These vesicles fluoresce brightly and are evident as fluorescent spots in these photographs. Photographs and studies of cytonemes were made with a Leica research or confocal microscope. To estimate the diameter of the cytonemes, A + C fragments were cultured for 1 hr, and CCD images were recorded in the green wavelength with a 1.4 numerical aperture objective. Cytoneme diameter was determined by comparison with images of Magnification Reference Standards obtained from Geller Microanalytical Laboratory. Epifluorescence microscopy was with a long-pass GFP Endow filter cube (Chroma). The rate of cytoneme growth was estimated by comparing photographs taken at several minute intervals at approximately 80 min after the incubation was initiated. This time is late in the growth period and reflects a low estimate, since the initial burst of growth appears to be considerably more rapid. The estimate we give is comparable to the growth rate of sea urchin thin filopodia (25 $\mu\text{m}/\text{min}$; Miller et al., 1995), but less than that of the acrosomal process of *Thyone* sperm (10 $\mu\text{m}/\text{s}$; Tilney and Inoue, 1982).

Drosophila Stocks

The P element insertion in the 3S line is on the X chromosome. It has been mapped to polytene band 7B and to the *brinker* gene (Minami et al., 1999). Homozygous mutant embryos were identified with the aid of *TKG* balancer chromosomes that express GFP (D. Casso et al., unpublished data). *bnl/bnl* or *bt/bt* embryos at the germband extended stage were identified by their lack of GFP expression from stocks of either *bnl/TKG* or *bt/TKG* flies.

Somatic Clones

Small clones expressing GFP were induced late in development in imaginal discs using the FLP/FRT technique (Xu and Rubin, 1993).

The genotype was *hsFLP, ux-abx>f>Gal4, β Gal/UAS-GFP*, and clone induction was by heat shock during the second larval instar. Discs were examined as above.

Growth of Cytonemes in Culture

Cytonemes growing from disc cells were observed with small pieces of discs that were dissected from third instar larvae. These fragments were placed between two glass coverslips in a drop of PBS or S2 medium and incubated at room temperature. Cytonemes were occasionally observed in solo cultures of C fragments, a result that we assume can be attributed to the presence of more outlying A or P cells in the dissected fragment. Growth in the presence of nocodazole or phalloidin-rhodamine was with A fragments and C fragments from wing discs, cultured in PBS under a coverslip. After 30 min, nocodazole (at 10 μM and 500 μM) or phalloidin-rhodamine (10 nM) was added under the coverslip. Cultures with nocodazole were continued for an additional 60 min.

Cultures with the *hh^{ts}* allele were made as above except that the larvae were incubated at either 18°C (permissive temperature) or 29°C (nonpermissive temperature) for 14–16 hr before the discs were isolated. Fragments were cultured for 1 hr at 29°C, and the growth of cytonemes was then assayed with either fluorescence or DIC optics.

Embryo suspensions were prepared by placing 2–3 germband extended embryos, whose vitelline membrane had been manually removed, in PBS on a microscope slide. Cells were gently dissociated under the weight of a coverslip, and cytonemes were observed after 45–60 min incubation using fluorescence or differential interference contrast optics.

S2 cells were transfected by standard calcium phosphate methods with 5 μg of DNA. Plasmids included Actin5C-GAL4 and equivalent amounts of either *UAS-dpp*, *UAS-Hh-N*, or *UAS-bnl* (Sutherland et al., 1996). After 2–3 days of culture, the cells were collected by centrifugation, and the cell pellet was placed on a glass coverslip and cocultured with tissue isolated from wing imaginal discs. Incubation was for 90 min.

Mouse culture limb bud cultures: GFP-expressing 10.5-day-old limb buds were isolated from GFP/GFP or GFP/+ mice. Non-GFP limb buds were isolated from +/+ littermates. A non-GFP forelimb bud tip was dissected and cocultured with a small piece of limb bud cells containing GFP in the various tissue fragment (apical ectodermal ridge, anterior forelimb, and zone of polarizing activity). Cocultures were placed on a coverslip, and after 50–60 min, cells were examined for cytonemes.

Acknowledgments

We thank Peter Lawrence for suggesting the name cytoneme and D. Casso for his suggestions of possible mechanisms by which cytonemes might calibrate distance. We thank M. Hoffmann for the *UAS-dpp* cDNA clone; M. Krasnow for the *bnl* cDNA clone, and *bnl* and *bt* fly lines; G. Martin for her suggestion to test the role of FGF; members of the Martin lab, M. Lewandoski and C. Chi, for help with the mouse experiments; J. Sedat for help with microscopy; S. Chan and D. Casso for isolation of the *3SGal4* line; members of the Kornberg lab for helpful discussions; and family and numerous colleagues for suggestions and help with the manuscript. This work was supported by an NIH Minority Postdoctoral Grant to F.-A. R.-W., and NIH and Merck Genome Research Institute grants to T. B. K.

Received April 6, 1999; revised April 30, 1999.

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