

PRIMER

Cytonemes as specialized signaling filopodia

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ABSTRACT

Development creates a vast array of forms and patterns with elegant economy, using a small vocabulary of pattern-generating proteins such as BMPs, FGFs and Hh in similar ways in many different contexts. Despite much theoretical and experimental work, the signaling mechanisms that disperse these morphogen signaling proteins remain controversial. Here, we review the conceptual background and evidence that establishes a fundamental and essential role for cytonemes as specialized filopodia that transport signaling proteins between signaling cells. This evidence suggests that cytoneme-mediated signaling is a dispersal mechanism that delivers signaling proteins directly at sites of cell-cell contact.

KEY WORDS: Cytoneme plasticity, Cytoneme transfer, *Drosophila*, Filopodia, Morphogen, Gradient

Introduction

The number of scholarly reviews on the topic of morphogen signaling published in the past two years exceeds the number of known morphogens; the reader is referred to excellent collections (Briscoe et al., 2010; Hill and Van Aelst, 2012) for in-depth treatments of its experimental and theoretical history. Morphogens are signaling proteins that are produced by, and released from, cells of a ‘developmental organizer’ (also known as a signaling center), and that disperse to direct the subsequent development of target cells. Their graded distribution across developmental fields is thought to embody the positional information that instructs neighboring cells to adopt particular fates. Studies in many systems show that cells in gradient fields respond to morphogen signaling proteins in a concentration-dependent manner.

Decapentaplegic (Dpp) signaling in the *Drosophila* wing imaginal disc is arguably the example of morphogen transport and signaling that is best understood. In the wing disc, Dpp is produced by a narrow band of cells that lies alongside the anterior/posterior compartment border at the disc midline (Posakony et al., 1990), and it forms broad concentration gradients across cells on both sides of the midline (Entchev et al., 2000; Teleman and Cohen, 2000). The responses of target cells vary in ways that correlate with both their distance from the Dpp source and the level of Dpp that they encounter (Lecuit and Cohen, 1998; Nellen et al., 1996), such that cells close to the source express downstream targets of Dpp signal transduction (e.g. *spalt*) that require levels of Dpp above a comparatively high threshold, whereas targets that require less Dpp for activation (e.g. *optomotor-blind*; *bifid* – FlyBase) are also expressed by cells at greater distances from the source where Dpp levels are lower. The correlations between the levels of Dpp, levels of Dpp signal transduction, expression of target genes, and the cell-autonomy of the requirement for the Dpp receptor Thickveins (Tkv)

has been interpreted as evidence that Dpp acts directly on client cells to elicit concentration-dependent responses.

The French Flag model of Wolpert (Wolpert, 1969) proposes that the positional value of a cell is established by a particular concentration of morphogen and that cells respond to different threshold concentrations. Although in principle this model can account for the Dpp-dependent domains of gene expression observed in the wing disc, a single morphogen concentration gradient may lack the necessary precision and reliability. For example, Wolpert has recently asserted that diffusible morphogen gradients are not a plausible mechanism for defining positional information because they lack precision: they are ‘too messy’ (Richardson, 2009). Additional inputs may be required, for instance from planar cell polarity (Kerszberg and Wolpert, 2007) or temporal dynamics (Nahmad and Lander, 2011). These ideas highlight the fact that our understanding of the molecular processes of pattern formation is fragmentary. What then do we know and what do we not know?

We know that morphogens are made in discrete locations by groups of cells, that they distribute across developmental fields to form concentration gradients, and that they act directly on client cells that are located at various distances from the morphogen-producing cells. We do not know how transcription and translation of morphogens are regulated or the degree to which these processes are subject to either positive or negative feedback. Most, or perhaps all, morphogens are post-translationally processed, but we do not know if processing is regulated, if processing and release are coordinated, or if processing is obligatory for release. We do not know the form of the protein that is released – whether free as a monomer, free as a multimer, or in a vesicle. We do not know how the expression of morphogen receptors is regulated and, although the components of most morphogen signal transduction systems have been identified, we do not understand how these systems may convey levels of morphogen receptor occupancy or how information from multiple pathways is integrated.

The fact that morphogens move across developmental fields does not imply or predict the mechanism by which they move. Diffusion is one possible mechanism and it has been a core tenet of almost every model of pattern-generating gradient formation. It is an unquestioned assumption in the models of Turing (Turing, 1952), Wolpert (Wolpert, 1969) and Meinhardt (Meinhardt, 1978), and Crick famously reported calculations showing that the diffusion of small organic molecules (300-500 Da) that are able to diffuse rapidly within and between cells can generate gradients within the time frame and over distances that would be required in known biological systems (Crick, 1970). Contemporary treatments have taken the chemical nature of signaling proteins into account, and have shown that the kinetics and distributions of morphogen in various systems are consistent with diffusion (e.g. Müller et al., 2012; Schwank et al., 2011; Yu et al., 2009; Zhou et al., 2012). Diffusion would seem to be the simplest mechanism: morphogen released from cells can take a random walk to locate and instruct surrounding cells. However, it is not the only possible mechanism,

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and showing that the dynamics and contours of morphogen gradients correlate with distributions that diffusion might generate (Zhou et al., 2012) does not establish causality. Because we know so little about how morphogen proteins are made and released, or how they are processed after uptake, it is problematic to choose between alternative mechanisms of dispersion based on steady-state distributions, on the contours of concentration gradients, or on levels of signal transduction in responding cells.

Recent findings, based on studies of Dpp and fibroblast growth factor (FGF) signaling in the *Drosophila* wing imaginal disc, provide visual and functional evidence for a direct delivery mechanism of morphogen dispersion. Here, we review this work and discuss it in the broader context of long distance signaling by both neurons and non-neuronal cells.

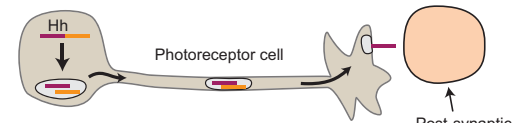
Contact-mediated signal dispersion

The direct delivery model of morphogen dispersion posits that morphogens can be transferred between cells at sites of cell-cell contact. This mechanism is based on specialized filopodia, termed cytonemes [cytoplasmic threads (Ramírez-Weber and Kornberg, 1999)], that extend between morphogen-producing and target cells, functioning as conduits for morphogen traffic. The direct delivery model involves cell-to-cell transfers in a way that ensures that signaling is specific to the cells that make direct contact; it is conceptually similar to signaling at neuronal synapses. Neurons signal by extending processes (axons and dendrites) that can reach across intervening cells to terminate at synaptic junctions. The cell bodies of the communicating cells may be far apart, but exchanges of neurotransmitters are focused to a synapse and the identities of the communicating cells can be precisely specified.

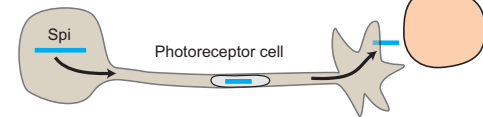
Neurons respond to protein signals as well as to neurotransmitters. Examples include neurotrophins, which are growth factors that bind to receptors at synapses and are taken up and transported to the neuronal cell body. Hedgehog (Hh), epidermal growth factors (EGFs) and Wingless (Wg) also signal at *Drosophila* neuronal synapses. Photoreceptor neurons in the developing retina, for example, synthesize Hh and EGF/Spitz (Spi) and release them at axonal termini (Fig. 1A,B), where they are picked up by post-synaptic neurons in the brain (Chu et al., 2006; Huang and Kunes, 1996; Yogev et al., 2010). The EGF ligand Gurken is a post-synaptic cue that regulates target selection at neuromuscular junctions (Naylor and DiAntonio, 2012). Wg signals at glutamatergic neuromuscular junctions in the embryo (Speese and Budnik, 2007), and experiments showing how Wg moves from pre- to post-synaptic cells are the only ones to unequivocally identify the secreted form of any morphogen signaling protein. Remarkably, Wg is not secreted in a soluble form, but moves between signaling and target cells in export vesicles (Fig. 1C). Wg concentrates in vesicles at the pre-synaptic boutons, and release of these vesicles carries Wg across the synaptic gap where it is taken up by the post-synaptic cell (Korkut et al., 2009). Wg transit from the producing cell to the receiving cell therefore combines one-dimensional transport along long cellular extensions and cell-cell transfer by a process that is confined to the tightly regulated environment of the synapse. This mechanism has little in common with the ‘sailor journey’ of a random walk over a long distance (Müller et al., 2013).

The direct delivery model of morphogen dispersion proposes that such movement along cell extensions, together with release and transfer at sites of contact, is a general mechanism that is also used by morphogen signaling proteins in non-neuronal contexts. In this model, morphogen signaling proteins travel between non-neuronal cells by a process that shares key features of Wg movement between pre- and post-synaptic cells.

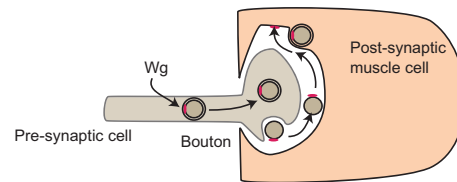
A Hh signaling by retinal neuron



B EGF signaling by retinal neuron



C Wg signaling at neuromuscular junction



D Cytoneme-mediated direct delivery in wing disc

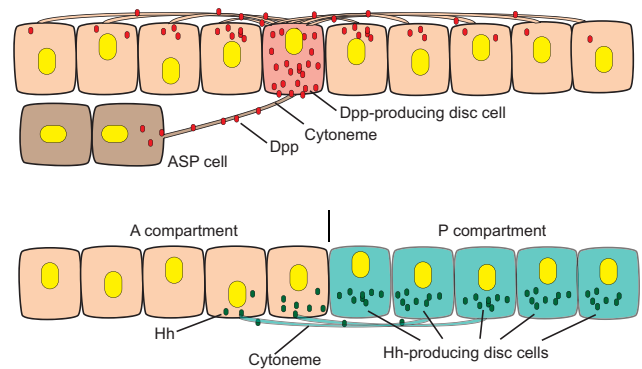


Fig. 1. Transfer of *Drosophila* signaling proteins at sites of direct cell-cell contact. Four contexts are illustrated in which signaling proteins transfer between producing and receiving cells at synapses. (A) In the developing visual system, the N-terminal fragment of processed Hedgehog (Hh, red bar) separates from the C-terminal fragment (orange bar) and is released to a post-synaptic neuron in the lamina after transport down a retinal axon (Chu et al., 2006; Huang and Kunes, 1996). (B) The *Drosophila* EGF-like protein Spitz (Spi, blue) moves by a similar mechanism in retinal neurons (Yogev et al., 2010). (C) *Drosophila* Wingless (Wg, red) is released in a vesicular form at developing neuromuscular synapses (Korkut et al., 2009). (D) Decapentaplegic (Dpp, red circles) moves from wing disc cells to air sac primordium (ASP) cells at synapses that form at cytoneme tips (Roy et al., 2014). Hh (green circles) moves from P compartment cells to cells across the compartment border along cytonemes (Callejo et al., 2011).

Cytonemes – specialized signaling filopodia

Filopodia are thin, actin-based protrusions that extend from cells. They have been observed in many types of cells and in many developmental contexts, and have been given many names – microspikes, pseudopods, thin filopodia (Miller et al., 1995), thick filopodia (McClay, 1999), gliopodia (Vasenkova et al., 2006), myopodia (Ritzenthaler et al., 2000), invadopodia (Chen, 1989), telopodes (Popescu and Faussone-Pellegrini, 2010), tunneling nanotubes (Rustom et al., 2004) and cytonemes (Ramírez-Weber and Kornberg, 1999). Although these protrusions share physical

properties – all are constructed with tight parallel bundles of actin filaments that assemble with actin-related and other cytoskeletal proteins – published descriptions suggest that these various filopodia and filopodia-like structures are not identical. Some are as thin as 0.1 μm in diameter, whereas others measure 0.4 μm ; some can be as short as 1 μm , whereas others extend more than 200 μm . Furthermore, despite having been observed more than 100 years ago, their roles have remained unproven because it has not been possible to selectively remove or inactivate them without compromising the integrity of the cells that produce them.

Filopodia have most often been associated with cell migration and wound healing (Fulga and Rørth, 2002; Izzard, 1974; Ridley et al., 2003; Wood et al., 2002) and with force generation (Locke, 1987; Sheetz et al., 1992; Vasioukhin et al., 2000). In addition, their dynamic behaviors in systems that support real-time observation have also suggested sensory roles as ‘antennae’ that probe the environment during processes such as vasculogenesis (Gerhardt et al., 2003; Lawson and Weinstein, 2002) and neurite pathfinding (Bentley and Toroian-Raymond, 1986; Dickson, 2002; Goodman, 1996; Lohmann and Bonhoeffer, 2008; Zheng et al., 1996). For example, the growth cones of developing axons have many filopodia that rapidly grow and retract, appearing to search the surrounding space for guidance cues (Bentley and Toroian-Raymond, 1986). Although these apparent sensory behaviors of endothelial and neurite filopodia have been attributed to gradients of diffusing chemotropic guidance molecules, there is no direct *in vivo* evidence for such gradients or for the dispersion of these guidance molecules by diffusion.

Studies of filopodia behavior have also suggested contact-dependent functionalities. Neuron pathfinding in grasshopper limbs appears to depend on contact between growth cone filopodia and particular guidepost cells (Sabry et al., 1991). Dynamic filopodia that make contact between interacting cells have also been observed in two contexts in *Drosophila* embryos: between muscle cells and neurons during motoneuron targeting and the formation of neuromuscular synapses (Ritzenthaler and Chiba, 2003; Ritzenthaler et al., 2000); and between neurons and glia in the central nervous system (Vasenkova et al., 2006). Dynamic filopodia also appear to be responsible for functional interactions between dendrites and axons in the developing mammalian hippocampus, where dendritic filopodia have lifetimes that range from minutes to hours and have tips that appear to make transient contacts (Lohmann and Bonhoeffer, 2008). Some of the contacts of the dendritic filopodia are more stable than others, leading to the idea that the filopodia mediate active signaling that discriminates among possible targets.

Filopodia with similar dynamic characteristics have also been observed in non-neuronal cells; indeed, studies of the filopodia made by primary mesenchyme cells of the sea urchin blastula were the first to note the dynamic nature of filopodia in live embryos (Gustafson and Wolpert, 1961). The intriguing behaviors of these filopodia led the authors to speculate that they play active roles as sensors of patterning information. Although direct evidence for the function of these filopodia is still lacking, data from subsequent studies are consistent with both structural (Hardin and Cheng, 1986) and sensory (Miller et al., 1995) roles. Most interesting and relevant to this discussion were parallels that were drawn between the ‘thin filopodia’ of the primary mesenchyme cells and the filopodia of neuronal growth cones (Miller et al., 1995). These two filopodia types have similar diameters and extension and retraction rates, and the observed responses to perturbations are consistent with roles for both as sensory implements that extend the reach of cells into the surrounding space.

Cytonemes, which are specialized types of signaling filopodia, were first noted as long cellular extensions that protrude from

Drosophila wing imaginal disc cells (Ramírez-Weber and Kornberg, 1999). These cytonemes have the defining characteristic that, irrespective of cell location in the wing primordium, they orient uniformly toward the disc midline where the morphogen signaling protein Dpp is expressed. The presence of such long filopodia that extend between wing disc morphogen-receiving cells and the Dpp-expressing cells at the midline suggested an alternative possibility to diffusion-based models of Dpp dispersion – that physical contacts are sites at which Dpp transfers from signal-producing cells to their targets (Ramírez-Weber and Kornberg, 1999). Dpp distributes across the wing disc and can be detected more than 20 cell diameters away from the cells that make it (e.g. Kicheva et al., 2007). Although diffusion-based models propose that Dpp finds receptors to bind on target cells by a random walk (either through or around intervening cells) after it is secreted by expressing cells (Kicheva et al., 2007; Schwank et al., 2011; Zhou et al., 2012), the cytoneme model proposes that Dpp is secreted only at sites of cytoneme contacts and that dispersion takes place on or in cytonemes. The process by which cytonemes identify appropriate targets is not known and might involve a random walk; the point is that signaling at cytoneme contacts does not.

Cytonemes in *Drosophila*

Although detecting cytonemes can be technically challenging and has been problematic (see Box 1), cytonemes and similar cell extensions that are associated with cell-cell signaling have been found in various *Drosophila* tissues (Table 1). Several types can be distinguished based on shape, location and composition, and their properties suggest that each signal transduction system employs a distinct and specific set. There are, for example, cytonemes that are distinctive to the eye and wing discs and to tracheal cells. In the eye disc, cytonemes on the apical surface of columnar epithelial cells orient to either the morphogenetic furrow (MF) or the equator. The EGF receptor (EGFR) is present in motile puncta in the cytonemes that orient to the MF (Fig. 2A) where Spi/EGF is expressed (Roy et al., 2011). In the wing disc, the Dpp receptor Tkv is present in motile puncta in cytonemes that orient to the disc midline (Fig. 2B) where Dpp is expressed (Hsiung et al., 2005). The attributes and behaviors of these cytonemes suggest that, via their motile receptor-containing puncta, they transport Dpp across the disc; the cytoneme tips directly contact the midline cells and the cytonemes appear to be directed only to cells that express Dpp. They are found tracking along the apical surface of the columnar epithelium and do not survive fixation (Fig. 1D). By contrast, basal cytonemes appear

Box 1. Technical challenges to the detection of cytonemes

Cytonemes can be difficult to detect because most do not retain their normal shape after fixation, and most are stunted by exposure to fixatives. Antibody staining is therefore problematic. Cytonemes can be marked by fluorescent proteins such as cytoplasmic GFP and membrane-tethered GFP, or with fluorescent proteins that are fused to cytoneme components, such as actin, and signaling protein receptors. Fluorescent images can be obtained at high magnification in unfixed tissue marked in these ways, although the resulting fluorescence from the 100–200 nm thick extensions is low and difficult to detect if fluorescence from surrounding tissue is significant. In addition, fluorescence quenching, phototoxicity and issues with *ex vivo* culture limit or prohibit real-time viewing, and most cytonemes do not lie in a single focal plane, which further complicates imaging. These technical issues explain why cytonemes were not detected prior to the development of systems for the robust expression of fluorescent proteins and why their characterization has accelerated as these systems have improved.

Table 1. Cell extensions associated with signaling in *Drosophila*

Tissue	Description	Proposed function	References
Imaginal discs			
Wing disc	Apical, wing primordium; oriented to Dpp- and Wg-expressing cells	Dpp, Wg reception	Ramírez-Weber and Kornberg, 1999; Hsiung et al., 2005; Roy et al., 2011
	Basal, wing primordium; oriented to/from the midline	Hh dispersion	Roy et al., 2011; Bilioni et al., 2013; Callejo et al., 2011
	Basal, notum primordium	Notch/Delta lateral inhibition	Cohen et al., 2010
Leg disc	Myoblasts, no apparent directional bias	?	Roy et al., 2011
	Apical between columnar and peripodial cells	?	Demontis and Dahmann, 2007
	Planar polarized protrusions of socket cells	EGF delivery	Peng et al., 2012
Eye disc	Columnar to adepithelial cells; adepithelial along proximodistal axis	?	Poodry and Schneiderman, 1970
	Apical, oriented to equator and morphogenetic furrow	EGF reception	Roy et al., 2011
Pupal discs	Oriented posteriorly from morphogenetic furrow	Scabrous transport, ommatidial rotation	Chou and Chien, 2002
	Apical between columnar and peripodial cells	?	Gibson and Schubiger, 2000; Cho et al., 2000
Wing disc-associated tracheal cells			
	Leading edge of spreading imaginal cells	Epithelial fusion	Martin Blanco, 2000
Lymph gland			
	Basal ASP and transverse connective, oriented toward Dpp- and FGF-expressing disc cells	Dpp, FGF reception	Sato and Kornberg, 2002; Roy et al., 2011
Ovary			
Germ cells	Posterior signaling center niche	Hh delivery	Mandal et al., 2007; Krzemien et al., 2007; Tokusumi et al., 2012
	Cap cells, toward adjacent escort cells	Hh delivery	Rojas-Rios et al., 2011
Egg chamber	Border cells	Oriented to EGF, VEGF sources, border cell migration	Fulga and Rørth, 2002
	Nurse cells	Border cell migration	Goode and Perrimon, 1997
Embryo			
	Muscle cell myopodia	Motoneuron innervation	Ritzenthaler et al., 2000; Kohsaka and Nose, 2009
	Leading edge, epithelia	Dorsal closure, wound healing	Jacinto et al., 2000; Millard and Martin, 2008

more resistant to fixation (Callejo et al., 2011), and their shape and orientation differ slightly from the apical cytonemes (Hsiung et al., 2005; Roy et al., 2011). Studies characterizing these basal cytonemes under conditions of overexpression of Interference hedgehog [Ihogg, a transmembrane Hh-binding protein (Yao et al., 2006)] suggest that they can deliver Hh from posterior cells to the midline; cells at the midline also extend basal cytonemes toward the posterior (Bilioni et al., 2013). In the tracheal air sac primordium (ASP), which is a branch that associates with the wing disc, cytonemes extend from the basal surface of the tracheal epithelium (Fig. 1D). Some ASP cytonemes contain Tkv but not FGF receptor (FGFR), whereas others contain FGFR but not Tkv (Fig. 2C). However, cytonemes containing both receptors have not been detected (Roy et al., 2011). This differential segregation of receptors to different cytonemes shows that cells can respond to multiple signals by extending cytonemes that are specific to different signal transduction systems. It is not known whether neuronal growth cone filopodia are similarly specialized and segregate receptors differentially.

In addition to specifically receiving or delivering signals and to having different receptors, cytonemes exhibit extreme variations in length. Some in the wing disc extend to over 80 μm . Observations of such long cytonemes have been rare compared with shorter forms, but their presence shows that cytonemes can reach across the full expanse of cells that respond to Dpp. However, we assume that the role of cytonemes is not a function of length. Rather, they are conduits that move signaling proteins between cells, functioning in a similar manner whether the cells are immediate or distant neighbors; the transfer of signaling proteins is governed by the nature of the contacts that cytoneme tips make and is independent

of the length of the cytoneme that presents the tip to a target cell. The rate and efficiency with which signaling proteins move along a cytoneme will thus influence the distribution of these signaling proteins across the region between the morphogen-producing and -receiving cells, but release and transfer are controlled at sites of contact.

Cytonemes in vertebrates

Filopodia with properties consistent with signaling functionality have been noted in many vertebrate cell types. In transformed mammalian cell lines, filopodia are associated with EGF (Lidke et al., 2005) and FGF (Koizumi et al., 2012) signal transduction. They have been observed extending from *Xenopus* XTC cells (Holzer et al., 2012), from B cells (Gupta and DeFranco, 2003), from mast cells induced by chemokines (Fifadara et al., 2010), and from neutrophils induced by nitric oxide and 4-bromophenacyl bromide (Galkina et al., 2009) or aggregation of the A3-adenosine receptor (Corriden et al., 2013). In developmental contexts, filopodia are associated with patterning of pigment cells in zebrafish (Inaba et al., 2012) and with transport of Hh in the chick limb bud (Sanders et al., 2013). Although only the studies with cultured cells obtained direct evidence for a role in signaling (Koizumi et al., 2012; Lidke et al., 2005), the properties described for the filopodia in the other contexts are consistent with the idea that these organelles either deliver or take up signals that move between cells.

Cytoneme plasticity and specificity

Signaling mechanisms in pattern formation must accommodate changing environments as growth and morphogenesis transform developing organs. In the wing disc, for instance, although wing disc

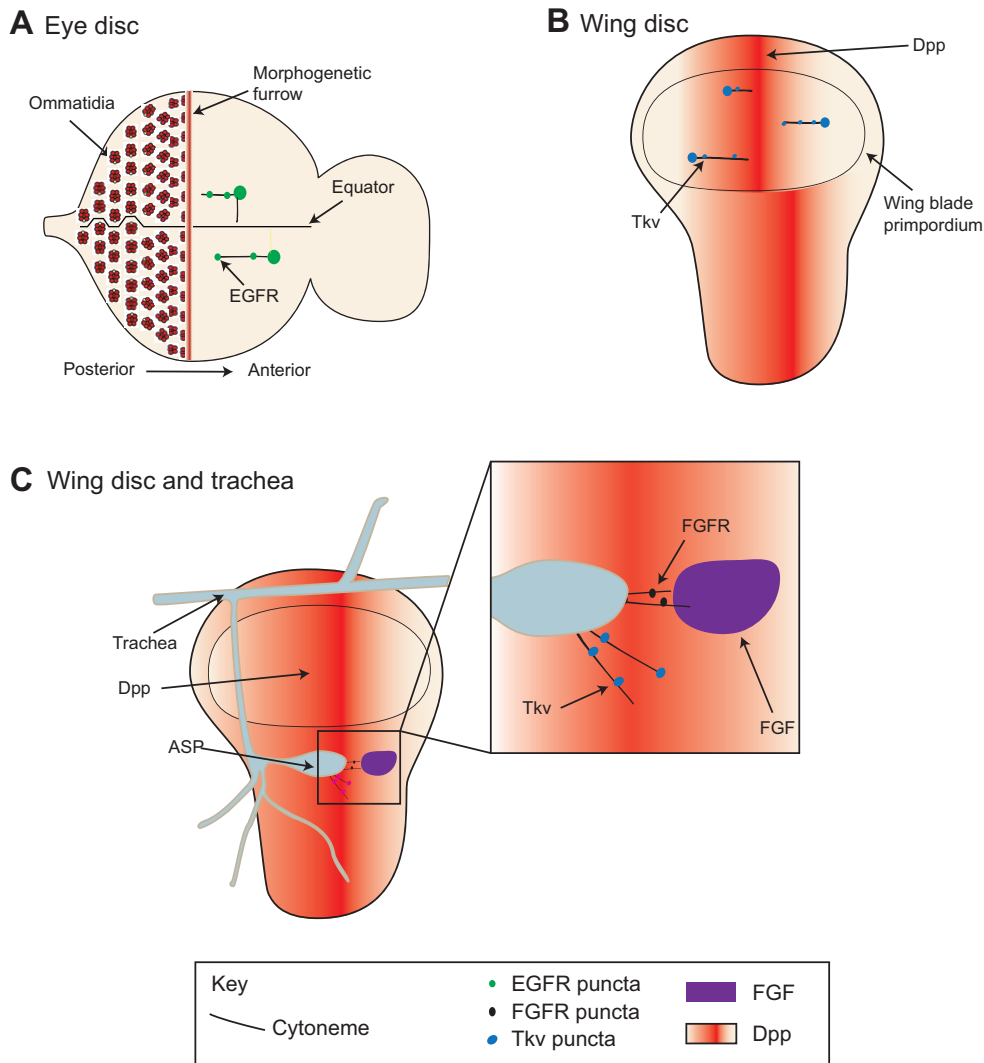


Fig. 2. Cytomes of the eye and wing imaginal discs. (A) An eye disc in which the morphogenetic furrow has progressed anteriorly across part of the eye field, highlighting two types of cytoneme (black lines) extending from cells anterior to the furrow: (1) cytonemes oriented toward the equator; and (2) cytonemes oriented toward the furrow and populated with EGFR-containing puncta (green dots). (B) A wing disc with the concentration gradient of Dpp depicted (red) showing cells in the wing blade primordium that extend cytonemes toward the Dpp-expressing cells and that are populated with Tkv-containing puncta (blue dots). (C) A wing disc and its associated trachea with ASP cells depicted with: (1) cytonemes that extend from the ASP tip to FGF-expressing disc cells (purple) and that are populated with FGFR-containing puncta (black dots); and (2) cytonemes that extend from the medial region to Dpp-expressing disc cells and are populated with Tkv-containing puncta (blue dots).

development initiates in the embryo, the dorsoventral signaling center appears and becomes operational much later during larval development, and cells at the FGF signaling center that induces and directs development of the ASP first activate FGF expression at the late second or early third instar. Yet every study of cytonemes reports that their distribution reflects the signaling landscape, regardless of stage or context. For example, cells that activate Dpp signal transduction extend cytonemes to the closest cells that produce Dpp in every experimental condition that has been examined. These observations suggest that cells produce or stabilize specific cytonemes to adapt to their specific signaling landscape.

This specificity is evident in the wing disc, where cells in the wing blade primordium direct Tkv-containing cytonemes toward the Dpp-producing cells at the disc midline to which they are closest. Specificity is also evident in the ASP cells that direct Tkv-containing cytonemes toward nearby Dpp-producing cells of the wing disc while also directing FGFR-containing cytonemes toward wing disc cells that express FGF. Furthermore, cells in the eye disc direct EGFR-containing cytonemes toward Spi/EGF-producing cells of the MF.

In the normal condition, cells extend cytonemes to the usual signaling centers and cytoneme distribution is highly reproducible. However, experimental conditions that change the location of the signaling cells also change cytoneme distributions. This plasticity is

evident, for example, in wing discs with reduced levels of Dpp (e.g. *dpp^{ts}*): the apical cytonemes that normally orient to the Dpp-expressing cells do not direct uniformly to the midline in discs with reduced Dpp function (Hsiung et al., 2005). In wing discs with uniform overexpression of Dpp (e.g. *hs-dpp*), the apical cytonemes are short and extend outward without apparent directional bias. Furthermore, in wing discs with small groups of cells that ectopically express Dpp, cytonemes emanating from cells in the vicinity of these groups orient toward the ectopic sources (Roy et al., 2011). Cytonemes in eye discs behave similarly with respect to changes in Spi/EGF expression, and ASP cytonemes behave similarly after changes to Dpp and FGF expression (Roy et al., 2011; Sato and Kornberg, 2002). These behaviors show that changes to the placement of cells that express signaling proteins are reflected in altered distributions of cytonemes and that, in all conditions that have been examined, cytonemes appear to link producing and receiving cells.

These properties do not imply a mechanism that targets cytonemes from morphogen-receiving cells to sources of signaling protein, but highlight the correlation between oriented cytonemes and the cells that produce signaling proteins. The observed plasticity of cytonemes indicates that their distributions and contacts are not permanent and suggests that the steady-state distributions that have been observed depend upon active signaling to form or maintain

contacts. A requirement for signaling is certainly consistent with the absence of cytonemes in conditions in which signal transduction is blocked, either after ectopic expression of a dominant-negative receptor or if expression of the signaling protein is reduced or eliminated. Moreover, their distributions and plasticity reveal their specificity for a particular signaling protein. As described above, eye disc cytonemes change after uniform overexpression of Spi/EGF, but they do not change after uniform overexpression of Dpp or Hh. Similarly, ASP cytonemes change after ectopic expression of FGF and wing disc cytonemes change after ectopic expression of Dpp, but these cytonemes do not change after uniform overexpression of Spi/EGF or Hh. These behaviors imply that stable contacts require contributions from both the signal-producing and -receiving cells.

Cytonemes transfer signaling proteins from producing to target cells

The presence of the Tkv, Breathless (Btl) and EGF receptors in separate, specific cytonemes suggests that each of these cytoneme subtypes mediates the movement of Dpp, FGF and Spi/EGF, respectively. Supporting evidence is of three kinds.

First, cells that activate the signal transduction pathways for these signaling proteins also extend cytonemes that contain the cognate receptor. This correlation holds for the cells in the wing disc that activate Dpp signal transduction and extend Tkv-containing cytonemes toward Dpp-expressing cells, for the cells of the eye disc that activate EGF signal transduction and extend EGFR-containing cytonemes toward the furrow, for the cells at the tip of the ASP that activate the FGF signal transduction and extend FGFR-containing cytonemes, and for cells elsewhere in the ASP that activate Dpp signal transduction and extend Tkv-containing cytonemes.

Second, studies in several other systems have reported that signal transduction is associated with cell-cell contacts both for cells that are far apart and for cells separated by short distances. For example, Hh that is involved in juxtacrine signaling in the *Drosophila* germline stem cell niche is localized in cytonemes that extend from Hh-expressing cells (Rojas-Ríos et al., 2012) and that are less than 1 μm long. In the *Drosophila* leg mechanosensory organ, Spi/EGF is produced in a socket cell and induces a particular neighbor to adopt a bract cell fate; polarized protrusions that originate from the socket cell appear to target EGF signaling to the particular precursor cell (Peng et al., 2012). Filopodia-mediated contacts between cells that are not immediate neighbors have also been implicated in Notch and Scabrous-dependent signaling that pattern the bristles of the adult thorax (Cohen et al., 2010; Renaud and Simpson, 2001). These varied structures reveal that these specialized filopodia may be tailored to fulfill specific roles, but their common functionality supports the model of contact-mediated long distance signaling.

Third, cytonemes link wing disc and ASP cells and are required for signaling. Dpp that is in transit between the wing disc and the ASP colocalizes with the Tkv receptor in puncta at cytoneme synapses and moves along cytonemes (Roy et al., 2014). These cytonemes extend up to 40 μm from the ASP and make contact with Dpp-expressing disc cells. The activated state of the Tkv that colocalizes Dpp in the puncta and the retrograde movement of the puncta indicate that the Dpp is receptor bound and that the cytonemes transport Dpp from the disc to the ASP. Evidence supporting this functional role for the ASP cytonemes as signaling filopodia is both molecular and genetic. The ASP cells that extend the cytonemes containing receptor-bound Dpp are able to activate Dpp signal transduction, and ASP cells that are genetically compromised for *diaphanous* (which encodes a formin), *shibire* (which encodes a dynamin), Neuroglian (an L1-CAM) or *capricious*

(which encodes a leucine-rich repeat transmembrane protein) fail to make normal cytonemes or cytoneme synapses and are signaling deficient (Roy et al., 2014). Because these mutant conditions did not compromise the capacity of cells with defective cytonemes to activate signal transduction cell-autonomously, these experiments establish that cytoneme-mediated transport is required for signaling.

The capricious (caps) phenotype has particularly interesting implications. Although ectopic expression of Caps^{DN} reduced Dpp signaling in the ASP, the number and distribution of ASP cytonemes was not detectably altered. However, the number of contacts with disc cells, as well as Dpp uptake and Dpp signaling, were severely reduced. The apparent lack of Dpp trafficking by cytonemes that do not contact disc cells is consistent with the observation that only cytonemes that contact Dpp-expressing disc cells have puncta that contain both Dpp and activated Tkv. These results indicate that contact is essential for transport and signaling, and imply that if free, diffusible Dpp is present in the ASP environs, it cannot induce signal transduction and cannot be transferred to or along cytonemes that do not make direct cell-cell contact.

How, then, might these findings be reconciled with experiments showing that morphogen that has been applied exogenously can activate signal transduction? If a neurotransmitter is provided exogenously from a pipet, a neuron can initiate a receptor-dependent action potential, but this response does not imply that neurotransmitter released at a synapse from a pre-synaptic cell is physiologically irrelevant or that an extra-synaptic source of neurotransmitter is physiologically relevant. In this context, the response of a tissue culture cell to a morphogen signaling protein in the culture medium is also irrelevant to the mechanism that exposes a cell to the same signaling protein *in vivo*. Receptors on the cell surface activate signal transduction in response to ligand binding irrespective of the original source of ligand. Applying this line of reasoning to the experiment with the ASP cells with defective cytonemes, the observation that these cells neither take up nor respond to Dpp means that their Tkv receptors are not exposed to Dpp. Therefore, the Dpp in these experiments that is produced by the disc cells (which are normal) is either not released or, if it is, does not engage and activate Tkv.

Perspectives

Cytoneme-mediated morphogen dispersion is now supported by strong observational and genetic evidence. Although many questions remain unanswered – for instance, how cytonemes locate their targets and how gradients form – it is wrong to dismiss the importance or relevance of cytonemes because our understanding of them is incomplete. Indeed, the only prediction that we make with confidence is that the answers will be both fascinating and unexpected.

Cells generate specific outputs in response to instructions that they receive, perhaps doing so by integrating quantitative effects of multiple signals. Although it is possible that the process that presents signals to the recipient cell (i.e. the mechanism of dispersion) is also involved in signal transduction, cytonemes might simply function as conduits, leaving signal transduction and signal integration to other machinery in the cell body. For example, studies in cultured human adenocarcinoma cells reported that retrograde transport carries receptor-bound EGF along filopodia and that receptor endocytosis occurs at the base of the filopodia (Lidke et al., 2005). The colocalization of Dpp and activated Tkv in motile puncta that move along cytonemes toward the cell bodies of ASP cells (Roy et al., 2014) is also consistent with the idea that signal transduction does not initiate until receptor-bound protein has been delivered.

Cytonemes may nevertheless be selective conduits. Development is characterized by complex geometries, by multiple signaling centers in developmental fields that may be closely juxtaposed, and by a limited and shared vocabulary of signaling proteins. We have previously argued that these issues make a random walk mechanism of dispersion unlikely (Kornberg, 2012; Kornberg and Guha, 2007), and that it is more likely that signaling proteins are targeted to particular cells. A key attribute of such a direct delivery mechanism is that the release and uptake of signaling proteins can be limited to the sites where specific contacts are made. Possible models for target selection include sensing an extracellular gradient for navigation to its source, influences of other cues (such as planar cell polarity), and random search. Although gradient sensing cannot be discounted, observations of the dynamic behavior of filopodia in sea urchin gastrula (Gustafson and Wolpert, 1961; McClay, 1999; Miller et al., 1995), zebrafish embryos (Lawson and Weinstein, 2002), the chick limb bud (Sanders et al., 2013), growth cone filopodia (Bentley and Toroian-Raymond, 1986) and *Drosophila* embryos (Jacinto et al., 2000; Ribeiro et al., 2002; Ritzenthaler et al., 2000) are consistent with a random search mechanism.

How might cytoneme-mediated dispersion generate concentration gradients? We can suggest two possible mechanisms: one in which the efficiency of transport is inversely proportional to the length of a cytoneme (caused, perhaps, by either inefficient trafficking or the limited half-life of an activated state); and one in which the contact frequency of dynamic cytonemes is inversely related to cell-cell distance and in which transport flux is directly proportional to contact frequency. However, we know too little about the cell biology and regulation of these structures to distinguish between these or other possible mechanisms, or to predict how they might provide the requisite 'robustness' (Eldar et al., 2003) or scaling (Ben-Zvi et al., 2011) of gradients. The absence of cytonemes under conditions of ectopic expression of dominant-negative receptor suggests that cytoneme stability and cytoneme contact depend upon functionalities in both the cell that extends a cytoneme as well as its target. Moreover, proteins that regulate signaling levels are known, and there are ample precedents for activity-based potentiation.

Steady-state images can accurately describe spatial distributions, but if a morphogen that is present between two cells is cytoneme associated, then we cannot infer the mechanism if we do not know the rate of cytoneme elongation and retraction, the stability of contacts or the flux of morphogen along a cytoneme, or which steps are rate limiting in the process of transfer at the cytoneme tip, transport along the cytoneme and delivery to the cell body. Although the contours of morphogen gradients have been described, most images have lacked sufficient resolution to distinguish whether the morphogen protein that is present between the cell bodies of signal-producing and -receiving cells is attached to plasma membranes, is bound within the extracellular matrix, is free in the extracellular fluid, or is tethered to a cytoneme. Cytonemes are difficult to image, and because treatments that destroy them are prone to leaving exosome-like remnants behind, histology that does not preserve or resolve cytonemes cannot distinguish between 'free' and cytoneme-associated protein. Nor can fractionation distinguish protein bound to extracellular components from proteins tethered to cytonemes that are easily broken. Finally, studies with antibodies that detect extracellular protein do not resolve between 'free' protein and protein bound to the external surface of a cell or to its cytoneme. Therefore, whereas imaging can establish that signaling proteins associate with cytonemes, images that lack the resolution or sensitivity to resolve cytonemes cannot ascertain their precise localization. It is the precise state of a morphogen when it is

between producing and receiving cells that distinguishes between diffusion- and cytoneme-based dispersion. Future work will inform us how the processes that regulate cytoneme-mediated transport fulfill Wolpert's call for more reliable and quantitative mechanisms of cell-cell signaling.

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