LETTERS

# Dependence of *Drosophila* wing imaginal disc cytonemes on Decapentaplegic

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**The anterior/posterior (A/P) and dorsal/ventral (D/V) compartment borders that subdivide the wing imaginal discs of *Drosophila* third instar larvae are each associated with a developmental organizer. Decapentaplegic (Dpp), a member of the transforming growth factor-β (TGF-β) superfamily, embodies the activity of the A/P organizer. It is produced at the A/P organizer and distributes in a gradient of decreasing concentration to regulate target genes, functioning non-autonomously to regulate growth and patterning of both the anterior and posterior compartments[1–3]. Wingless (Wg) is produced at the D/V organizer and embodies its activity[4,5]. The mechanisms that distribute Dpp and Wg are not known, but proposed mechanisms include extracellular diffusion[6], successive transfers between neighbouring cells[7,8], vesicle-mediated movement[9], and direct transfer via cytonemes[10]. Cytonemes are actin-based filopodial extensions that have been found to orient towards the A/P organizer from outlying cells. Here we show that in the wing disc, cytonemes orient towards both the A/P and D/V organizers, and that their presence and orientation correlates with Dpp signalling. We also show that the Dpp receptor, Thick-veins (Tkv), is present in punctae that move along cytonemes. These observations are consistent with a role for cytonemes in signal transduction.**

Cytonemes appear as fluorescent strands emanating from the apical surface of disc cells that express green fluorescent protein (GFP)[10]. Using standard epifluorescence microscopy, cytonemes are visible only if neighbouring cells have low background fluorescence, only in unfixed discs, and only if they extend in a single optical plane. The contour of the apical surface of the notum primordium is rather flat (see Supplementary Fig. 1), and cytonemes can be imaged in this region in discs that are suspended in liquid. However, the wing pouch primordium is convex, and cytonemes can only be imaged in discs that have been slightly flattened. The fragile nature of disc cells requires that physical and osmotic insults be minimized, and the methods we have developed to image wing cytonemes avoid rupture, delamination and other responses to injury (see Methods). Typical examples of successful preparations with flattened discs are shown in Fig. 1.

In the micrograph shown in Fig. 1b, small clones (averaging 10–15 cells) express CD8–GFP and are visible in a speckled pattern. High-magnification views of clones in this and similar discs reveal cytonemes extending outwards from some, but not all clones. On the basis of the presence or absence of cytonemes and on the orientation of cytonemes, three regions of the disc can be distinguished. In the wing blade primordium, approximately 20% of the clones extended cytonemes oriented towards either the A/P (Fig. 1c, i) or D/V compartment borders (Fig. 1h, i). More than 95% of these clones had cytonemes oriented towards one of the two borders, and <5% had cytonemes orientated towards both. Figure 1 shows a clone imaged at two adjacent optical planes: A/P-oriented cytonemes are visible in the more apical plane and D/V-oriented cytonemes are visible in the more basal plane of the clone. We have not been able to establish whether all cells extend cytonemes, whether cells can extend more than one cytoneme, or whether a single cell can extend

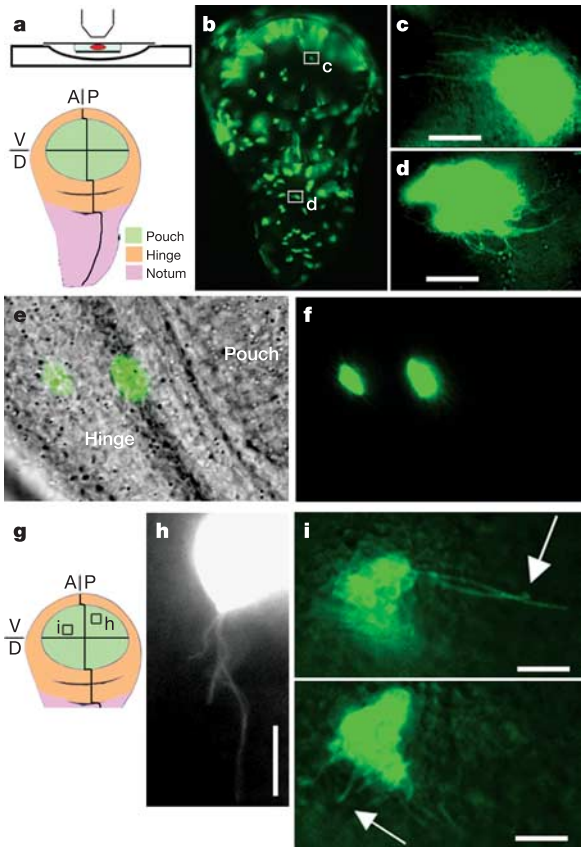


**Figure 1 | Cytoneme profile. a**, Schematic of the mounting method (top panel) and drawing of a wing disc with A/P (vertical black line) and D/V (horizontal black line) borders marked (bottom panel). **b**–**d**, Disc with randomly induced clones expressing CD8–GFP (**b**), with cytonemes visible at the higher magnifications (**c**, **d**). Wing pouch cytonemes are A/P-oriented whereas notum cytonemes are not. **e**, **f**, Cells labelled by clones in the hinge region do not extend cytonemes. **g**, **h**, Drawing showing a clone in the ventral-posterior region of the pouch with D/V-oriented cytonemes (**h**). **i**, Consecutive optical sections showing a clone in the ventral-anterior region of the pouch (marked in **g**) extending both A/P (top panel) and D/V (bottom panel) cytonemes. All clones are marked with CD8–GFP. Scale bars, 10 μm.

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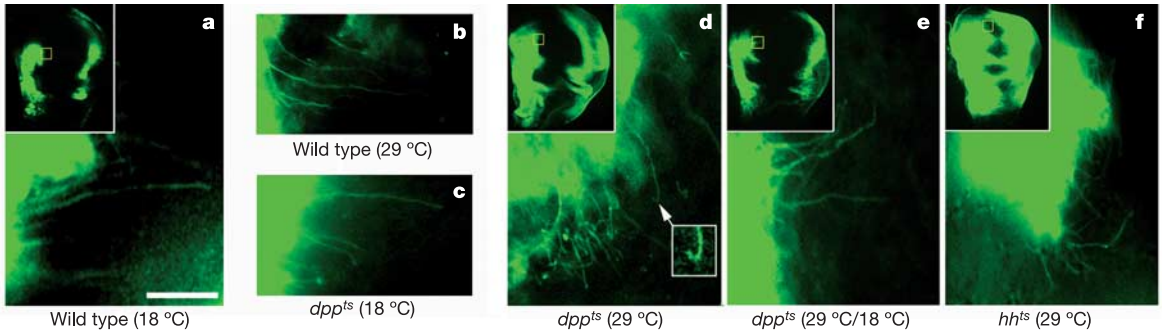


**Figure 2 | Dpp maintains A/P cytoneme orientation.** **a**, *brinker* expression in the wing disc (inset) and A/P-oriented cytonemes extending from *brk*-expressing cells in a wild-type background. **b**, **c**, Controls showing that the orientation and distribution of cytonemes is normal in wild-type wing discs after incubation at 29 °C (**b**) and in $dpp^{ts}$ mutants at 18 °C (**c**). **d**, In $dpp^{ts}$ mutant discs, the *brk* expression domain expands towards the centre after 5 h at 29 °C. Cytonemes increase in number and orient irregularly, some with 'hooked' tips (enlargement in **d**). **e**, The phenotype returns to nearly wild-type after a 3-h recovery period. **f**, $hh^{ts}$ mimics the effects of $dpp^{ts}$. All images show *brk >CD8–GFP* in either a wild-type (**a**, **b**) or mutant background (**c**–**f**). Scale bar in **a**, 10 μm. All figures shown at the same scale.

cytonemes towards both axes. A/P cytonemes as long as 80.2 μm have been recorded; the average length in these preparations is 20.8 μm. D/V cytonemes were shorter, averaging 8.8 μm (Supplementary Table 1).

Clones in the notum primordium radiated cytonemes in all directions, without a consistent bias towards either the A/P or D/V axes of the disc. Cytonemes associated with notum clones averaged 7.4 μm in length, almost 65% shorter than A/P cytonemes in the wing primordium. Unlike cells in the wing and notum primordia, cells in the hinge/pleural primordium did not extend cytonemes. We examined these hinge/pleural cells by expressing GFP in clones (Fig. 1e) and by using an enhancer trap expressed in the hinge region (not shown), but did not observe cell extensions in either case.

Although Dpp is essential for cell survival in the notum[11], there is no indication that the A/P compartment border in the notum has an associated organizing centre, and it is ambiguous whether Dpp functions as a morphogen in the notum region. Dpp is apparently not required for either growth or cell survival in the hinge/pleural region[12]. Thus, directional cytonemes are present in the wing primordium (where Dpp functions as a morphogen), cytonemes are present and 'omni-directional' in the notum (where Dpp may function only to support cell proliferation), and cytonemes are absent in the hinge/pleural region, where Dpp function appears not to be required. These three distinct cell types—cells with A/P- or D/V-oriented cytonemes, cells with cytonemes lacking a directional bias, or cells without cytonemes—could each be imaged in a restricted and defined region of the same disc.

We tested whether the shape and distribution of cytonemes in wing discs correlates with the presence of Dpp. First, when we reduced Dpp levels at the A/P organizer using temperature-sensitive mutants of either *dpp* ($dpp^{ts}$) or *hedgehog* ($hh^{ts}$) (*dpp* expression depends upon Hh signalling), cytonemes in the wing primordium were affected. At the permissive temperature (18 °C) in mutant discs, or at either the permissive or non-permissive (29 °C) temperatures in normal discs, cytonemes emanating from cells at the lateral flanks of discs oriented towards the A/P organizer (Fig. 2a–c). After incubation at 29 °C, however, cytonemes in $hh^{ts}$ and $dpp^{ts}$ mutant discs were more numerous (>twofold), and were not uniformly oriented towards the A/P organizer region (Fig. 2d, f). In these mutant discs, we observed curved and bent cytonemes, and cytonemes crossing over each other. Cytonemes with such shapes were never observed under normal conditions, or if mutant larvae were returned to the permissive temperature after a period of incubation at 29 °C (Fig. 2e).

Second, to test whether Dpp is sufficient for cytoneme induction, we imaged cytonemes in discs in which Dpp was expressed ubiquitously. In control discs, cells projected cytonemes towards A/P and D/V axes only (Fig. 3a), but in discs with heat-shock-induced Dpp (hs-*dpp*) >50% of the clones projected cytonemes outwards in all directions (Fig. 3b). These cytonemes were significantly shorter than those in untreated discs, averaging about 10.6 μm in length. Even more striking were the cells in the hinge domain, which normally do not extend cytonemes. Under conditions of ubiquitous Dpp expression, cells in the hinge domain extended cytonemes in apparently random orientations (Fig. 3c). Our ability to image cytonemes is limited to preparations in which discs have been extracted from larvae and the cytonemes are static, but their varied appearance under the conditions we have tested illustrates that they are dynamic *in vivo*. Although we favour a model in which they extend from cells in random directions but become stabilized when functional contacts are made with signalling cells, we cannot exclude the possibility that their directionality is directly influenced by extracellular cues.

Third, we monitored the distribution of the Dpp receptor Tkv, as a Tkv–GFP fusion protein[13]. When expressed in the lateral flanks of wing discs, most of the fluorescence was localized to the plasma membrane of expressing cells. However, bright, motile punctae were also present in more central regions, as far as 30 μm from the edge of the expression domain (Fig. 4a). These punctae were motile, moving in both anterograde and retrograde directions, and some images clearly revealed their association with cytonemes (Fig. 4b, see Supplementary Video). Trafficking of these punctae was approximately 5–7 μm $s^{-1}$ (Fig. 4c), a rate consistent with measured rates of vesicular movement on actin filaments[14]. The resolution of these studies could not establish whether they were inside or on cytonemes.

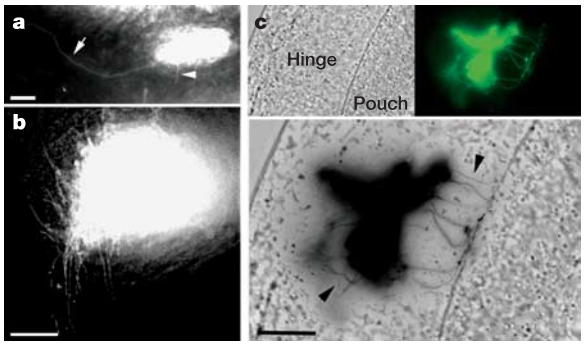


**Figure 3 | Dpp overexpression induces cytoneme extensions.** **a**–**c**, Cells in an hs-*dpp* background show normal A/P (arrow) and D/V (arrowhead) cytoneme orientation at 18 °C (**a**), but cells extend cytonemes in all directions after heat-shock (**b**), even in the hinge region, where cells normally do not extend cytonemes (black arrowheads in **c**, single channels shown above the merged image). Scale bars, 10 μm. Clones are marked with CD8–GFP.

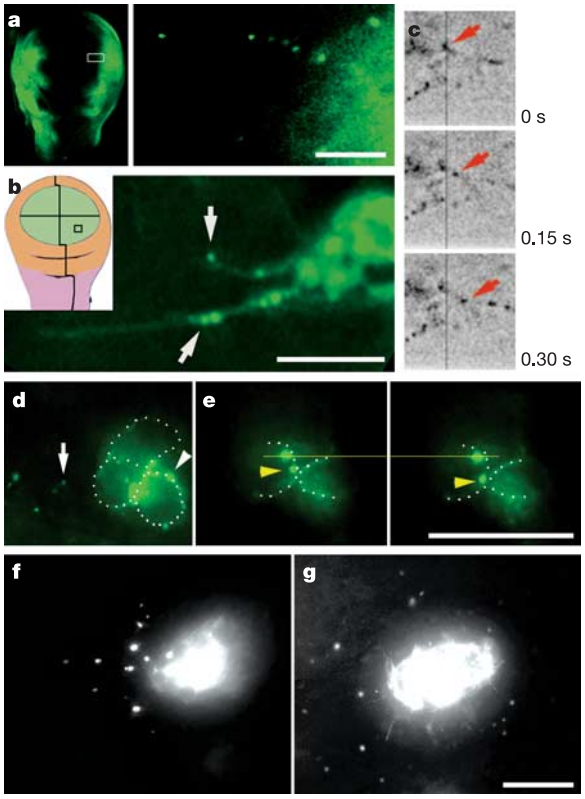


**Figure 4 | Cytonemes contain motile Tkv punctae.** **a**, *brk > Tkv–GFP* shows tracks of Tkv–GFP oriented towards the A/P border. **b**, Tkv–GFP punctae (arrows) can be seen localized to A/P cytonemes in Tkv–GFP clones. **c**, A motile Tkv–GFP puncta (red arrows) moving along a cytoneme. Vertical line shown as a reference point. Time shown in seconds. **d**, Control with dimethylsulphoxide shows no effect on the distribution of punctae both away from the cell bodies (arrow; cells outlined with white dots) and on the cell surface (arrowhead). **e**, Cytochalasin D reduces the number of Tkv–GFP punctae distributed at a distance from the cell bodies, but does not reduce or arrest punctae on the cell surface. Yellow arrowhead in left and right panels indicates a translocating Tkv–GFP puncta; horizontal yellow line shown as a reference point. **f**, **g**, Tkv–GFP in hs-*dpp* discs with (**f**, as in Fig. 3) and without (**g**) heat-shock. A/P border is to the left; images taken at the same scale. All scale bars, 10 μm.

We plotted the distribution of Tkv–GFP punctae around clones in discs with normal expression of Dpp or with ubiquitous Dpp expression. In normal discs, Tkv–GFP punctae were polarized in the direction of the A/P border (Fig. 4f). In contrast, Tkv–GFP punctae in heat-shocked hs-*dpp* discs were more numerous and projected in various directions all around the circumference of the clones (Fig. 4g). As these patterns of Tkv–GFP punctae could be imaged in unflattened discs, we compared their distribution in both flattened and unflattened discs. No differences were detected between the two conditions with respect to either the total number of punctae, or to the distance from or position relative to the clones (Supplementary Table 2). This confirms that the slight flattening we use to image cytonemes does not generate or substantially alter these structures.

Our previous work has demonstrated that cytonemes bind phalloidin (a specific F-actin-binding protein) and can be labelled with an actin–GFP fusion protein, suggesting that cytonemes are actin-based[10]. To test whether cytonemes and the movement of Tkv–GFP punctae is actin-dependent, we treated discs containing clones of Tkv–GFP-expressing cells with cytochalasin D, an actin-binding drug. The number of Tkv–GFP punctae at a distance from the cell bodies was dramatically reduced in treated discs (reduction estimated to be >90%; Fig. 4d, e). Bright punctae were observed on the surface of cells expressing Tkv–GFP, and they appeared to move along the surface of the cells even in the presence of drug (Fig. 4e). In contrast, the bright punctate fluorescence distant from GFP-expressing cells was not motile. These observations suggest that cytonemes can function as vehicles for active, actin-based transport of receptors.

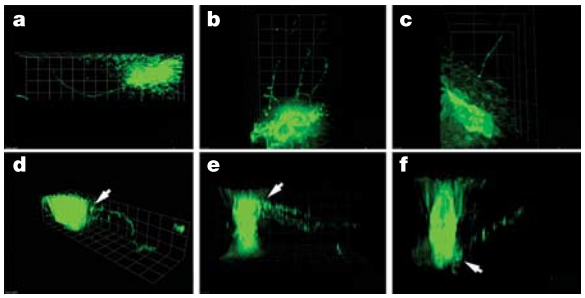


**Figure 5 | Distinct apical and basal cytonemes in the wing disc.** **a**, **d**, CD8–GFP-labelled clones. **b**, **e**, Tkv–GFP-labelled clones. **c**, **f**, Vav–GFP labelled clones. Top (**a**–**c**) and side (**d**–**f**) views of three-dimensional reconstructed images are shown. Apical side is up. Arrows indicate the point of cytoneme protrusions.

To better document the structure of cytonemes, we reconstructed optical sections of cytoneme-producing clones to render their three-dimensional structure. In such images, we observed that cytonemes labelled with CD8–GFP (Fig. 5a, d) as well as cytonemes containing Tkv–GFP punctae (Fig. 5b, e), extend from the apical surface of the disc columnar epithelial cells. In contrast, expression of a human guanine nucleotide exchange factor (GEF) protein, Vav–GFP, which has been shown to localize to filopodia in vertebrate cells[15], labels basal filopodia when expressed in wing disc cells (Fig. 5c, f). This preferential placement of proteins into different types of filopodial extensions indicates that apical and basal extensions are structurally distinct, it suggests that these cell extensions may be functionally distinct, and it implies the existence of a mechanism for sorting proteins to specific types of extensions.

Dpp is synthesized and secreted by a narrow stripe of 5–7 cells adjacent to the A/P compartment border in the wing primordium, and it distributes in a gradient of decreasing concentration that extends across the wing pouch[8,16]. A concentration gradient does not imply a mechanism for distribution; it is conceivable that cytonemes sense and respond to Dpp but do not ferry it. However, on the basis of the results presented here, we consider cytoneme-based transport to be an attractive possibility. As this work shows, the Dpp receptor Tkv is present in cytonemes, and the presence, orientation and shape of cytonemes in wing discs correlates with what is known about the different roles that Dpp has in the wing, notum and hinge primordia. Moreover, cytonemes change in response to conditions of Dpp gain-of-function and loss-of-function. These correlations are consistent with the idea that Dpp moves from its source in an oriented manner imposed by the directionality of these cellular extensions. Several recent studies reported have cellular extensions in *Drosophila* cells that correlate with signalling by Branchless (a *Drosophila* FGF, or fibroblast growth factor)[17–19], Notch[20] and Scabrous[21], extensions in spider cells that correlate with signalling by Dpp[22], and extensions in mammalian cells that correlate with signalling by epidermal growth factor (EGF)[23]. The widespread occurrence of cytonemes and cytoneme-like filopodia suggests that their role in long-distance signalling might be a general one, one that might permit selective signalling in ways that enable cells to regulate both release and uptake of signals.

## METHODS

**Fly stocks.** *brinker (brk)-Gal4* is an enhancer trap on the X chromosome[10]. *UAS-CD8–GFP* was a gift from L. Luo. $dpp^{ts}$ was created by placing $dpp^{4}$ and $dpp^{56}$ (obtained from F. Chanut) in-*trans* to each other. $hh^{ts}$ was obtained from the Bloomington stock centre (BL-1684), hs-*dpp* was from E. Bier. Random

CD8–GFP clones were generated by applying a heat shock to *hsFlp*; *abxubx > FRT > Gal4/UAS-CD8–GFP* to early third instar larvae for 12 min at 37 °C. In both $dpp^{ts}$ and $hh^{ts}$ experiments, larvae were grown at 18 °C, shifted to 29 °C and then immediately dissected and imaged, or were returned to the permissive temperature (18 °C) before dissection. *UAS-tkv–GFP* was created by inserting the enhanced (*E*)*GFP* coding sequence at the carboxy terminus of *tkv* and cloning into pUAST[24].

**Sample preparation method.** Imaginal discs were dissected and mounted in 1 × PBS buffer. Samples were placed on a coverslip, apical side down in a PBS droplet, and overlaid with a smaller coverslip to enhance flattening. The 'sandwich' was then turned upside-down, placed over the depression well of a slide and secured with halocarbon oil at the edges. Note that in the finished preparation, the samples, buffer and the smaller coverslip used for flattening, are all 'hanging' under the larger coverslip that contacts the objective lenses (see illustration in Fig. 1a). This allows the field to be scanned without damaging the sample, and minimizes additional compression of the samples. Cytochalasin D was used at 20 $\mu g\,ml^{-1}$ in Grace medium containing 5% dimethylsulphoxide[21]. Samples were incubated in the solution for 30 min on ice before imaging.

**Imaging.** Images were taken using an upright Zeiss Axioplan2 microscope equipped with a Cooke CCD camera, and were refined with deconvolution software (Slidebook v4.0, Intelligent Imaging Innovations). Tkv–GFP movement was captured with 100–153-ms exposure intervals. Time-lapse sequences and kinetic analysis of TKV–GFP trafficking were accomplished using Slidebook software. Confocal images were acquired with a Leica TCS laser scanning microscope and analysed using NIH Image J.

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**Supplementary Information** is linked to the online version of the paper at www.nature.com/nature.

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