

# Cytoneme-Mediated Contact-Dependent Transport of the *Drosophila* Decapentaplegic Signaling Protein

Sougata Roy, Hai Huang, Songmei Liu, Thomas B. Kornberg\*

**Introduction:** In multicellular organisms, morphogen signaling proteins move from “signaling centers” where they are produced to target cells whose growth and patterning they regulate. Whereas much progress has been made identifying and characterizing signaling proteins such as the transforming growth factor- $\beta$  family member Decapentaplegic (Dpp), which is produced in the *Drosophila* wing imaginal disc, the mechanisms that disperse signaling proteins remain controversial. We characterized Dpp signaling in a system in which cytonemes, a specialized type of filopodia implicated in long-distance signaling, could be imaged, and in which movement of signaling proteins and their receptors could be followed.

**Methods:** We expressed fluorescence-tagged forms of proteins that function in morphogen signaling to monitor Dpp in signal-producing cells, its receptor in signal-receiving cells, and proteins and cell structures that participate in trafficking of signaling proteins. Signaling was characterized in live, unfixed tissue as well as by immunohistochemistry, and under conditions of both gain- and loss-of-function genetics.

**Results:** Cells that received Dpp and activated Dpp signal transduction extended cytonemes that directly contacted Dpp-producing cells. The contacts were characterized by relative stability and membrane juxtaposition of less than 15 nm. Cytonemes that contained the Dpp receptor in motile puncta also contained Dpp taken up from Dpp-producing cells. In contrast, a different set of cytonemes that contacted fibroblast growth factor (FGF)-producing cells contained the FGF receptor but did not take up Dpp. The cytonemes were reduced in number and length in genetic loss-of-function conditions for *diaphanous*, which encodes a formin; for *neuroglian*, which encodes an L1-type cell adhesion molecule; and for *shibire*, which encodes a dynamin. Cytonemes were present in loss-of-function conditions for *capricious*, which encodes a leucine-rich repeat cell adhesion protein, but these cytonemes failed to contact Dpp-producing cells. Signaling was abrogated in all these conditions that created defective cytonemes, although the signal-producing cells were not compromised. The mutant conditions were not lethal to the affected cells, and the mutant cells retained competence to autocrine signaling.

**Discussion:** This work describes cytonemes that receive and transport signaling proteins from producing cells to target cells, and shows that cytoneme-mediated signal exchange is both contact-dependent and essential for Dpp signaling and normal development. Contact-mediated signal exchange and signaling are also the hallmarks of neurons—an analogy that extends to the functional requirements for the *diaphanous*, *neuroglian*, *shibire*, and *capricious* genes by both neurons and epithelial cells. Discoveries of cytonemes in many cell types and in many organisms suggest that contact-mediated signaling may be a general mechanism that is not unique to neurons.

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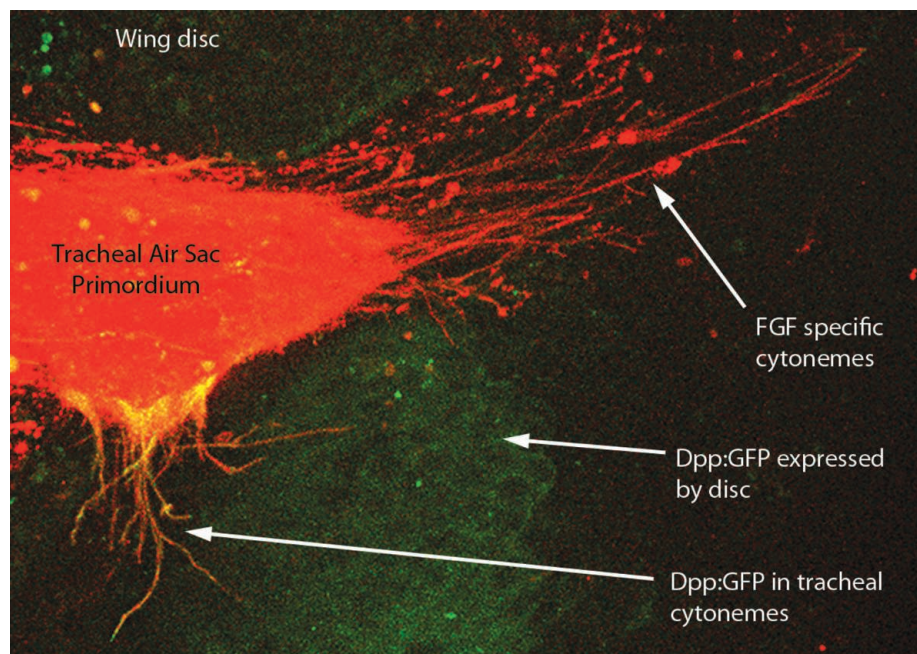
Fig. 6. ASP cytonemes require *Caps*.

## SUPPLEMENTARY MATERIALS

Figs. S1 to S3

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**Cytonemes take up and transport morphogens.** Micrograph showing a tracheal branch marked with mCherry overlying a wing disc expressing Dpp (tagged with green fluorescence). Cytonemes extend from the medial region of the branch to Dpp-expressing disc cells, and from the tip of the branch toward FGF-expressing disc cells. Dpp has been taken up and transported by the cytonemes that contact Dpp-expressing cells.

# Cytoneme-Mediated Contact-Dependent Transport of the *Drosophila* Decapentaplegic Signaling Protein

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Decapentaplegic (Dpp), a *Drosophila* morphogen signaling protein, transfers directly at synapses made at sites of contact between cells that produce Dpp and cytonemes that extend from recipient cells. The Dpp that cytonemes receive moves together with activated receptors toward the recipient cell body in motile puncta. Genetic loss-of-function conditions for *diaphanous*, *shibire*, *neuroglian*, and *capricious* perturbed cytonemes by reducing their number or only the synapses they make with cells they target, and reduced cytoneme-mediated transport of Dpp and Dpp signaling. These experiments provide direct evidence that cells use cytonemes to exchange signaling proteins, that cytoneme-based exchange is essential for signaling and normal development, and that morphogen distribution and signaling can be contact-dependent, requiring cytoneme synapses.

In many contexts during development, cell fate is determined by morphogen signaling proteins. The *Drosophila* wing imaginal disc, for instance, expresses the morphogen Decapentaplegic (Dpp), a transforming growth factor- $\beta$  family member that regulates the fate, proliferation, and patterning of its cells [reviewed in (1, 2)]. The disc expresses Dpp in a stripe of cells alongside the anterior/posterior (A/P) compartment border, and Dpp disperses across the disc to form exponential concentration gradients to either side that regulate target genes in adjacent cells in a concentration-dependent manner. Whereas the dispersion of Dpp across the disc and the functional importance of its concentration gradients are well established, the mechanism that moves Dpp from producing to target cells is not.

We tested the model that morphogens are transported along specialized signaling filopodia (cytonemes) that receive protein released at sites where producing and receiving cells contact each other (3). Cytonemes are on both the apical and the basal surfaces of wing disc cells. Apical cytonemes that orient toward Dpp-producing disc cells contain the Dpp receptor Thickveins (Tkv), and cytoneme shape, orientation, and distribution depend on the expression of Dpp (3–5). There are basal cytonemes that contain Hedgehog (Hh) and the Interference Hedgehog (Ihog) proteins (6, 7). Hh is also present in short cytonemes that extend from Hh-producing cells in the female germline stem cell niche (8). These correlations are suggestive, but they do not establish that cytonemes mediate transfers of signaling proteins from producing to target cells or that such transfers, if they occur, are required for signaling.

The wing disc has associated trachea whose development depends in part on signaling from the disc (9). Larval trachea form an interconnected network of oxygen-carrying tubes; one, the transverse connective (TC) of Tr2 is bound to the wing disc (Fig. 1A). During the third larval instar (L3), Branchless [the fly fibroblast growth factor (FGF)] produced by a group of disc cells induces a new branch, the air sac primordium (ASP), to grow from the TC (9). The ASP is juxtaposed to the basal surface of the wing disc columnar epithelium; it is a monolayered epithelial tube. At the late L3 stage, the ASP has many cytonemes that extend toward the disc (Fig. 1B). Cells at the ASP tip extend long ( $\geq 30$   $\mu$ m) cytonemes that contain the FGF receptor (FGFR) Breathless and appear to touch FGF-producing disc cells. The presence and orientation of these cytonemes are dependent on FGF (5, 9). The late L3 ASP also has shorter cytonemes that contain Tkv and that extend from its lateral flank toward Dpp-expressing disc cells (5).

In the wing disc, Dpp induces several changes in responding cells: induction of *Daughters against Dpp* (*Dad*) expression (10), increased phosphorylation of the Mothers against dpp protein (pMad) (11), and decreased *tkv* expression (11). Dpp signal transduction does not change expression of the other Dpp receptor subunit Punt (Put). Elevated *Dad* expression, increased pMad expression, and decreased *tkv* expression were observed in the ASP, presumably due to Dpp signaling, and their abundance indicates that Dpp signal transduction is probably higher in the lower layer cells that face the disc epithelium than in the cells that are further away in the upper layer (Fig. 1, C and D; fig. S1, A to D; and table S1). Put expression was uniform. Dpp expression was not detected in the TC or ASP (Fig. 1A and fig. S1E). These results show that Dpp signal transduction in the ASP inversely correlates

with distance from Dpp-expressing cells in the wing disc.

Overexpressing dominant negative forms of Tkv or Put, or *Dad* (which negatively regulates Dpp signaling), in the trachea generated abnormally shaped ASPs and reduced Dpp signaling in the ASP (Fig. 1E; fig. S1, F to H; and tables S2 and S3). Expression of *dppRNAi* in the wing disc generated similar phenotypes and reduced Dpp signaling (Fig. 1F and table S3), indicating that the wing disc is the source of the Dpp that activates signal transduction in the ASP, and establishing that Dpp signaling from the disc is essential for normal ASP development.

## ASP Cytonemes Receive Dpp from the Wing Disc

To investigate the basis for disc-dependent Dpp signaling in the ASP, we overexpressed an isoform of Dpp coupled to green fluorescent protein (Dpp:GFP) (12, 13) in the disc *dpp* expression domain (14). GFP fluorescence was detected both in the Dpp-expressing disc cells and in the ASP. Amounts of Dpp:GFP in the ASP were highest in the medial region of the ASP nearest the Dpp-expressing disc cells and in the lower layer (Figs. 1D and 2A and table S1), showing that Dpp:GFP produced by the wing disc distributed to the ASP in a manner that correlates with amounts of Dpp signal transduction (Fig. 1C and fig. S1, A to D). To examine the subcellular localization of marked Dpp in the ASP, we expressed Dpp coupled to mCherry fluorescent protein (Dpp:Cherry) (5) in the disc *dpp* expression domain, and Dpp signaling was monitored in unfixed, “live” preparations with a transgene that expresses nuclear-localized GFP (nGFP) under *Dad* control. Dpp:Cherry puncta were observed in multiple optical sections of ASP cells with strongly marked GFP-positive nuclei (Fig. 2, B and B’); the presence of Dpp:Cherry puncta at apical positions (Fig. 2B’’) indicated that Dpp:Cherry had likely been taken up from the disc by these ASP cells.

Whereas most tip cytonemes extended toward the region of the disc that expresses FGF (5, 9), some TC and lateral cytonemes extended toward Dpp-expressing disc cells (Fig. 1B). Expression of Tkv:GFP marked puncta in these cytonemes (Fig. 2C). To determine whether activated Tkv was present in cytonemes, we overexpressed a variant of Tkv (TIPF) that fluoresces only in the phosphorylated state and that has been used to monitor receptor activation for Dpp or bone morphogenetic protein (BMP) signaling (15). ASP cytonemes with bright fluorescent puncta were present under conditions of TIPF overexpression (Fig. 2D). Expression of Tkv:Cherry and TIPF together in the TC and ASP generated puncta with both green (TIPF) and red (Tkv:Cherry) fluorescence, indicating that Tkv in these puncta had been activated (Fig. 2, E and E’). We propose that the presence of activated Tkv indicates that these cytonemes had received Dpp. The presence of

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cytonemes with only red fluorescence suggests that not all the cytonemes had received Dpp.

To further validate and characterize Dpp reception, ASPs were marked with either CD8:Cherry (mCherry fused to the extracellular and transmembrane domains of the mouse lymphocyte protein CD8), Tkv:Cherry, or FGFR:Cherry, and Dpp:GFP was expressed in the disc *dpp* domain in a pulse during L3 (14). The ASP grows from the TC on the anterior side of the disc and extends posteriorly across the stripe of Dpp-expressing cells by late L3 (9) (Fig. 3A). At the “mid” or “late” stages, animals that expressed CD8:Cherry and Dpp:GFP had long ASP tip cytonemes marked with Cherry fluorescence that oriented toward FGF-expressing disc cells. These cytonemes had no apparent GFP fluorescence (Fig. 3B). Lateral ASP cytonemes that projected toward Dpp-expressing disc cells were also visible. These lateral cytonemes had both Cherry and GFP fluorescence (Fig. 3, B and B'), indicating that Dpp:GFP had been received by these cytonemes. Dpp:GFP in puncta “free” from either cells or cytonemes was not detected.

ASPs marked with Tkv:Cherry provided evidence that Dpp transport by cytonemes is associated with its receptor. Late-stage ASPs that expressed Tkv:Cherry had Dpp:GFP present in their medial region and in lateral cytonemes that extended from these cells, but there were few Tkv:Cherry-marked tip cytonemes, and Dpp:GFP was present in much lower amounts in the distal ASP cells (Fig. 3C). Some of the Dpp:GFPs present in the medial ASP cells were associated with Tkv puncta (Fig. 3C'). These images show that Dpp:GFP appears to move from the disc and are taken up by tracheal cells.

In mid-stage ASPs that expressed FGFR:Cherry and whose tip had not grown beyond the Dpp-expressing zone of the disc, FGFR:Cherry-marked tip cytonemes extended over Dpp-expressing disc cells toward the cells that expressed FGF (Fig. 3D). No Dpp:GFP puncta localized with the FGFR:Cherry-marked cytonemes. The absence of Dpp:GFP in the FGFR:Cherry-containing tip cytonemes is consistent with the localization of the FGFR and Tkv receptors to different cytonemes (5) and suggests that FGF and Dpp reception may be receptor-dependent and specific for cytonemes that contain FGFR or Tkv, respectively.

To better understand cytoneme-mediated movement of Dpp, we analyzed “early”- and mid-stage preparations that had Tkv:Cherry expressed in the trachea and Dpp:GFP expressed in the disc. Dpp source cells are distal to the ASP at these stages. Long, Tkv:Cherry-marked cytonemes extended toward Dpp-expressing disc cells (Fig. 3, E and F). These cytonemes contained motile puncta (movie S1). Some cytonemes had both Tkv:Cherry and Dpp:GFP fluorescence and had brightly fluorescent ends that localized with Dpp:GFP; these images suggest that these cytonemes contact Dpp-expressing disc cells. Not all cytonemes had both Tkv:Cherry and Dpp:GFP, suggesting that some, but not all, cytonemes had received Dpp:GFP.

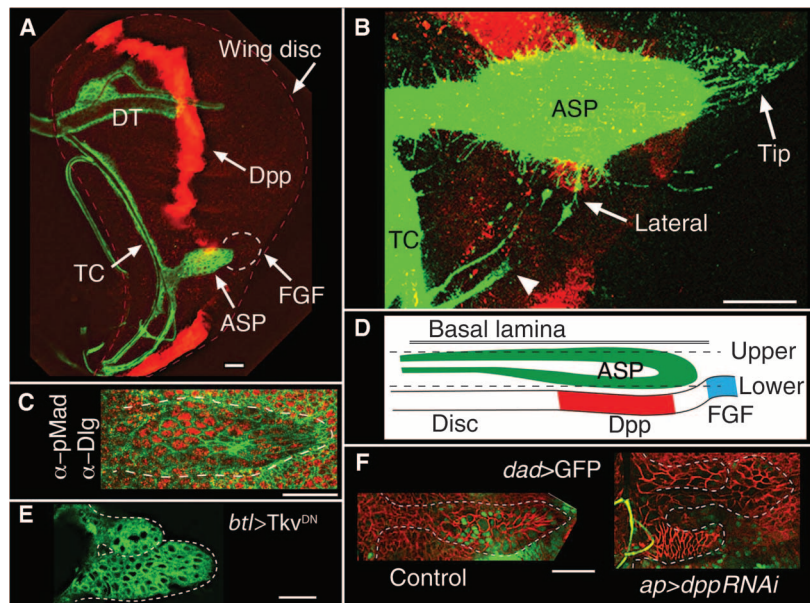
These images are consistent with the patterns of TIPF fluorescence (Fig. 2E). The presence of Dpp:GFP in tracheal cytonemes and the apparent contacts of cytonemes with Dpp-producing disc cells suggest that the Dpp:GFP may move from the disc to the tracheal cells by direct transfer at sites of cytoneme contact.

### Cytonemes Synapse with Wing Disc Cells

The cytoneme model of signaling protein dispersion posits that distant cells contact directly despite their physical separation. To probe the apparent contacts at higher resolution, we adapted the GRASP (GFP Reconstitution Across Synaptic Partner) technique, which was developed to image membrane contacts at neuronal synapses (16, 17). We expressed CD4:GFP<sup>1-10</sup> (a fragment of GFP that includes 10 strands of the GFP  $\beta$ -barrel photocenter fused as an extracellular postsynaptic to the transmembrane domain of the mouse lymphocyte protein CD4) and CD4:GFP<sup>11</sup> (a frag-

ment that includes the 11th strand of the GFP  $\beta$ -barrel). To image cytoneme contacts, the two parts of GFP were expressed separately in tracheal cells and in either FGF- or Dpp-expressing disc cells. These nonfluorescent GFP fragments generated fluorescence that localized specifically at the disc cells that expressed either FGF or Dpp (Fig. 4, A to C). Expression of mCherry-CAAX (CAAX is a plasma membrane-targeting motif) in the disc *dpp* domain revealed that GRASP fluorescence correlates with *dpp*-expressing cells (Fig. 4C). Fluorescence was separated from the ASP cells by up to 40  $\mu$ m (Fig. 4, A and B), the approximate length of the longest cytonemes that projected from the ASP toward disc cells, indicating that ASP and disc cells synapse even when separated. GFP fluorescence was not observed in animals that expressed only one of the fragments.

To show that the GRASP fluorescence was associated with cytoneme contacts, cytonemes



**Fig. 1. Dpp produced in the wing disc signals to disc-associated tracheal cells.** (A) Projection image of a third instar wing disc (outlined with dashed red line) showing disc-associated trachea (marked with green; membrane-tethered GFP) and Dpp-expressing disc cells (red, marked by antibody against LacZ that was expressed in the *dpp* domain). TC, DT (dorsal trunk), and ASP are labeled. Dotted circle indicates area of disc that expresses FGF. (B) Expression of CD8:RFP marks *dpp*-expressing disc cells (red); expression of CD8:GFP in trachea (*lexO*-CD8:GFP, *UAS*-CD8:RFP/+; *btl*-*LexA*/+; *dpp*-Gal4/+ ) marks cytonemes extending from ASP tip, from the lateral, medial region of the ASP, and from the TC (arrowhead), showing that some ASP and TC cytonemes orient toward Dpp-expressing cells. This plane of focus does not detect all *dpp*-expressing cells due to folds in the disc near the ASP organizer, but it did detect many *dpp*-expressing anterior cells that are in the plane of focus as “scattered” in the A compartment region between the ASP and TC. (C) Staining an ASP with antibody to pMAD (red) to show Dpp signaling in the medial region. Antibody to Dlg (discs large, green) marks cell outlines in ASP (bounded by white dashed line) and discs. (D) Cartoon of a sagittal ASP section depicting the position of the disc epithelium and basal lamina relative to the ASP in the late L3; dashed lines represent approximate locations of the upper and lower optical sections in all figures. (E) Overexpression of Tkv<sup>DN</sup> in trachea (*btl*-Gal4) generated bifurcated, abnormally shaped ASPs. (F) *dppRNAi* expression in the dorsal compartment of the disc (*ap*-Gal4 *tub*-Gal80<sup>ts</sup>) reduced *Dad* expression (*Dad*-nlsGFP, green) in disc-associated trachea (right panel) compared to control (*ap*-Gal4 *tub*-Gal80<sup>ts</sup> *Dad*-nlsGFP) ASP (left panel); abnormal ASP growths are indicated by white dotted lines; cells are marked with  $\alpha$ -Dlg staining (red); and both panels show lower layer of ASP. Orientation of discs in all figures: anterior, left; dorsal, down. Conditions of Gal80<sup>ts</sup> inactivation for (E) and (F) are described in table S1 (14). Scale bars, 30  $\mu$ m.

were marked independently of the GRASP GFP fragments by expression of mCherry-CAAX or Tkv:Cherry. Fluorescence of reconstituted GFP was mostly at or near cytoneme tips that contacted source cells (Fig. 4, A' and B'). Tkv:Cherry fluorescence had a punctal distribution in these cytonemes and was also present at contact sites (Fig. 4B'). An estimate of the size of the CD4 domains (diameter,  $\sim 65$  Å) (18, 19) and of the linkers that join CD4 to the GFP fragments suggests that the apposition of a cytoneme tip with a target cell at a synapse is less than 20 nm. This distance is comparable to neuronal and immune synapses, and because GFP photocenter maturation is not instantaneous (20), the GRASP fluorescence indicates that cytonemes can make relatively stable contacts with target cells.

The proximity of the tubular ASP and the disc varies along the ASP proximodistal axis (1.5

to 10  $\mu\text{m}$ ), and the anatomies of the two epithelia are complex (Fig. 4, D and D'). The ASP cells that overlie Dpp-expressing disc cells are in close apposition, yet in this region, cytonemes emanated from both the ASP (Fig. 4E) and the disc (Fig. 4F). The ASP cytonemes in this region were short ( $\leq 10$   $\mu\text{m}$ ); the disc cytonemes were as long as 30  $\mu\text{m}$ , and many had bright bulbous tips at apparent points of contact with ASP cells. GRASP marked the contacts between the lower layer of the ASP and the disc (Fig. 4G), but did not resolve the relative contribution of the ASP and disc cytonemes.

In the wing pouch primordium of the wing disc, Dpp-dependent cytonemes on the apical cell surfaces orient toward the stripe of Dpp-expressing cells at the A/P developmental organizer and may ferry Dpp from the A/P organizer to cells as far away as the disc flanks (3–5). We

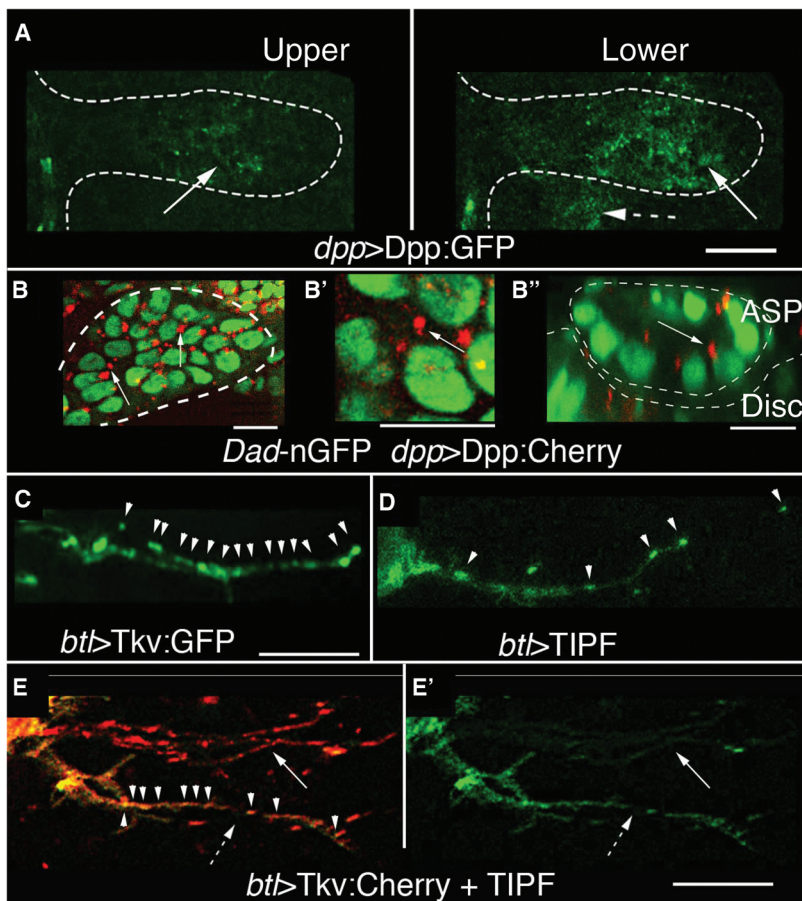
applied GRASP to image contacts between the wing disc A/P organizer and flank cells by expressing the GFP fragments at the A/P organizer and in flank cells (Fig. 4H). In these discs, GFP fluorescence was observed in the region of the organizer (Fig. 4H'), in contrast to discs that expressed only one of the complementing fragments (Fig. 4H''). This pattern of GFP reconstitution suggests that cytonemes may extend from the cells at the disc flanks to synapse with cells of the A/P organizer.

### Dpp Signaling in the ASP Requires Cytoneme-Mediated Transport

We identified four genes that are required for ASP morphogenesis and for cytoneme function: *diaphanous* (*dia*), *shibire* (*shi*), *neuroglian* (*nrg*), and *capricious* (*caps*). Mutant loss-of-function conditions were induced selectively in trachea during the L3 stage (14), and mutant ASPs were abnormal or duplicated at variable expressivity and penetrance (table S2 and fig. S1); we show and describe ASPs that were most normal in appearance. Wing discs in these experiments were not mutant, and wing disc development appeared normal.

The formin *Dia* is an actin nucleation factor (21) whose activated form localizes to the tips of filopodia (22). When *Dia:GFP* and activated *Dia:GFP* were expressed in the ASP, *Dia:GFP* was mostly in the cell body and was present at low levels in cytonemes, but activated *Dia:GFP* was prominent in most cytonemes and localized to cytoneme tips (Fig. 5A). The distribution of activated *Dia* indicates that cytoneme tips may be sites of actin nucleation. To examine the role of *Dia*, we expressed *diaRNAi* in the ASP during the L3 stage. In  $>85\%$  of the animals ( $n = 26$ ), growth of the ASP was decreased and ASP morphogenesis was abnormal (for example, fig. S1J). The number of cytonemes was also decreased, and many of the cytonemes that extended from mutant ASPs were abnormally short and had blunt tips (Fig. 5, B to E), and Dpp signal transduction (*Dad-GFP* expression and pMad abundance) was decreased (Fig. 5, F and G, and table S3). We did not detect changes to cell shape, number of dividing cells, or number of dying cells in mutant ASPs (fig. S2, A and B). Thus, *Dia* appears to be required by the ASP to make cytonemes, and the defective cytonemes that are made in the absence of normal *Dia* function are incapable of mediating Dpp signaling from the disc.

We expressed a conditional mutant of *shibire* (fruit fly dynamin; *shi<sup>ts1</sup>*) (24) together with CD8:GFP in the trachea and compared  $\alpha$ -pMad staining as well as the number and length of cytonemes in ASPs that were isolated from larvae that had been incubated at either permissive (18°C) or restrictive (30°C) temperature (Fig. 5H). Dynamin is a multimer (25, 26), and under non-permissive conditions, the *Shi<sup>ts1</sup>* protein functions as a dominant negative (24). Control larvae subjected to 3 hours at 30°C did not change the



**Fig. 2. The ASP takes up Dpp, and ASP cytonemes contain activated Tkv receptor.** (A) Dpp:GFP expressed in the disc *dpp* domain (*dpp-LexA lexO-Dpp:GFP*, dashed arrow) is present (arrows) in the upper and lower ASP layers in this unfixed preparation. ASP is outlined by white dotted lines. (B to B'') Dpp:Cherry expressed in the disc *dpp* domain (*dpp-Gal4/UAS-Dpp:Cherry*, *Dad-nGFP/tub-Gal80<sup>ts</sup>*) was detected as intracellular puncta (arrows) in ASP cells that also induce *Dad* expression. ASP outline is marked by white line [(B) and (B'), sagittal sections; (B''), transverse section]. (C) Expression of Tkv:GFP (*btl-Gal4 UAS-Tkv:GFP*) marks puncta (arrowheads) in ASP cytoneme. (D) Expression of TIFP (*btl-Gal4 UAS-TIFP*) marks puncta in ASP cytoneme. (E and E') TIFP (green) and Tkv:Cherry fluorescence (*btl-Gal4/UAS-Tkv:Cherry*; *tub-Gal80<sup>ts</sup>/UAS-TIFP*) colocalizes (arrowheads) in puncta in some, but not all, ASP cytonemes. Arrow, cytoneme with Tkv:Cherry only; dashed arrow, cytoneme with both TIFP and Tkv:Cherry; left panel, merge; right panel, TIFP only. *Gal80<sup>ts</sup>* inactivation for (B) and (E) was for 6 to 8 hours in mid L3, followed by incubation at 25°C for 6 to 12 hours. Scale bars, 10  $\mu\text{m}$ , except for (A), 30  $\mu\text{m}$ .

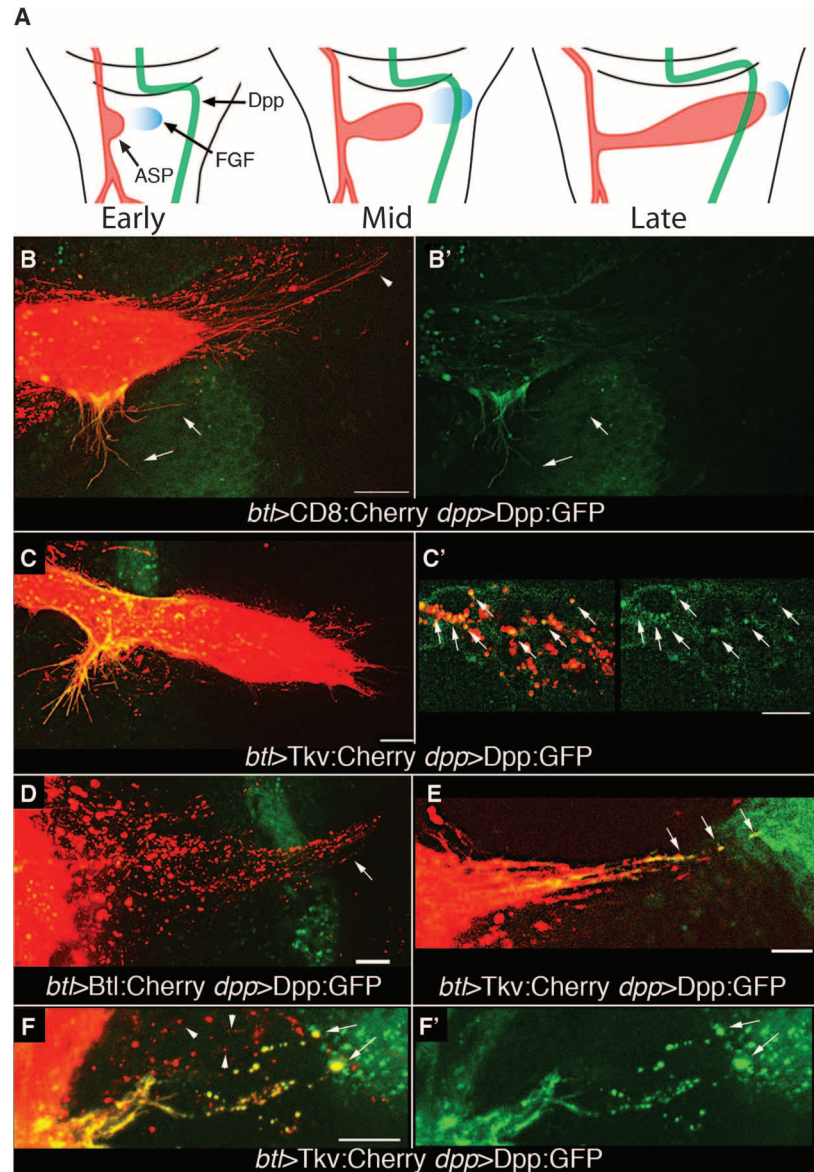
number of “short” (<25  $\mu$ m) or “long” (>25  $\mu$ m) ASP cytonemes ( $\sim$ 4.4 and  $\sim$ 4.9% reduction, respectively) or reduce amounts of pMad ( $\sim$ 7%). However, *shi<sup>ts1</sup>* larvae subjected to 30°C had decreased cytoneme numbers and pMad abundance (Fig. S1I and tables S3 and S4). The number of long cytonemes present after 30 min at 30°C was less than 10% of that in control experiments; numbers of short cytonemes also declined after 30 min at 30°C. Reductions in numbers of short cytonemes and amounts of pMad became more severe over time intervals of up to 3 hours. A 2-hour heat pulse and a 1-hour incubation at 20°C partially restored both long and short cytonemes (14), but the ASP morphology was not normal. Indeed, duplicated, abnormally shaped ASPs were produced when a 24-hour incubation at 20°C followed a 1-hour heat pulse (fig. S1I). Adults that developed at 20°C after a 2-hour heat pulse appeared to have normal morphology, and we did not examine the structure or function of their dorsal air sacs. Thus, *Shi* inactivation was not lethal in the cells of the ASP; the consequences of *Shi* inactivation on ASP development were partially reversible; and the effects on cytonemes preceded the reduction in signaling (as revealed by amounts of pMad).

To distinguish whether ASPs that are deficient for *dia* or *shi* expression failed to activate Dpp signal transduction because they did not receive Dpp from the wing disc or were incapable of initiating a response, we expressed Dpp:Cherry directly in ASPs with the *btl-Gal4* driver (14). Ectopic Dpp induced pMad in ASPs with reduced *dia* or *Shi* function (fig. S3, A and B). Thus, conditions that reduced *dia* expression or inactivated *Shi* did not abrogate the ability of ASP cells to respond to Dpp, and blocking cytoneme-mediated uptake of Dpp from the disc appears to be the most likely cause of the signaling deficits.

Cytonemes were also defective in loss-of-function conditions for *nrg* and *caps*, both of which encode putative cell adhesion transmembrane proteins. *Nrg* is an L1-type cell adhesion molecule implicated in the development and stability of neuronal synapses (27). Although fluorescence of an in-frame protein trap *Nrg*:GFP fusion protein was detected in the ASP, ASP cytonemes could not be resolved because of “background” expression in the wing disc. However, overexpression of *Nrg*:GFP in the ASP revealed that *Nrg* distributes in the ASP cytonemes and concentrates at the cytoneme tips (Fig. 5J). Expression of *nrgRNAi* reduced the number of both tip and lateral cytonemes (Fig. 5K and table S5), abrogated *Dad*-GFP expression and dpERK (diphospho-extracellular signal-regulated kinase) staining (Fig. 5, L and M), and caused growth of abnormal, duplicated ASP lobes (fig. S1K). Expression of *nrgRNAi* had no apparent effect on cell shape or the number or distribution of dividing or dying cells (fig. S2, C and D). Expression of Dpp:Cherry together with *nrgRNAi* in the ASP restored Dpp signaling (fig. S3C), indicating that *nrg*-deficient ASP cells can activate Dpp signal transduction.

We identified *caps* in an enhancer trap screen for genes that are expressed in the ASP (14) (fig. S2, E and F). *Caps*:GFP that was expressed in the trachea was detected in ASP cytonemes and

concentrated at the tips (Fig. 6A). *Caps* and its paralog *Tartan* (*Trn*) have extracellular domains contribute partially redundant functions to the for-



**Fig. 3. Tkv-containing cytonemes transport Dpp.** (A) Drawings of three third instar stages depict growth and development of the ASP (red) relative to wing disc cells expressing Dpp (green) and FGF (blue). (B and B') Expression of CD8:Cherry in the ASP and Dpp:GFP in the *dpp* domain of the disc (*btl-Gal4 UAS-CD8:Cherry dpp-LHG/lexO-Dpp:GFP*) marks the ASP and ASP cytonemes (red) and *dpp*-expressing disc cells (green). GFP fluorescence is in the lateral ASP cytonemes (arrows) and in the lower medial region of ASP, but not in the tip of ASP cytonemes (arrowhead). Left panel, merge; right panel, GFP. (C and C') Expression of *Tkv:Cherry* in the ASP and Dpp:GFP in the *dpp* domain of the disc (*btl-Gal4/UAS-Tkv:Cherry; dpp-LHG/lexO-Dpp:GFP*) marks the ASP and lateral ASP cytonemes (red), but few tip cytonemes; lateral *Tkv*-containing ASP cytonemes and the medial region of the ASP have received Dpp:GFP (green) (C). Dpp:GFP and *Tkv:Cherry* colocalize in puncta in ASP cells (C', arrows). (D) *Btl:Cherry* expressed in ASP and Dpp:GFP in the *dpp* domain of the disc (*btl-Gal4/UAS-Btl:Cherry dpp-LHG/lexO-Dpp:GFP*) marks puncta in the ASP tip cytonemes (arrow) that project beyond Dpp-expressing disc cells (green); no localization of *Btl:Cherry* with Dpp:GFP was apparent in tip cytonemes. (E, F, and F') Only cytonemes marked with *Tkv:Cherry* that appear to contact Dpp:GFP-expressing disc cells (*btl-Gal4 UAS-Tkv:Cherry; dpp-LHG/lexO-Dpp:GFP*) have GFP fluorescence in puncta and at their tips (arrows). Cytonemes that do not appear to make contact do not have GFP fluorescence at their tips or in their *Tkv*-containing puncta (F, arrowheads) lack GFP fluorescence. (F) merge; (F') Dpp:GFP. Animals were raised at 18°C to minimize transgene expression and were incubated at 22° to 25°C for 12 to 16 hours before analysis. Scale bars, 10  $\mu$ m.

mation of compartment boundaries of the wing disc (28). *caps* mutants do not mediate selection of synaptic partners normally (29–32), and Caps protein localizes at filopodia tips during partner recognition and synapse stabilization (30). We observed similar types of effects on ASP cytonemes.

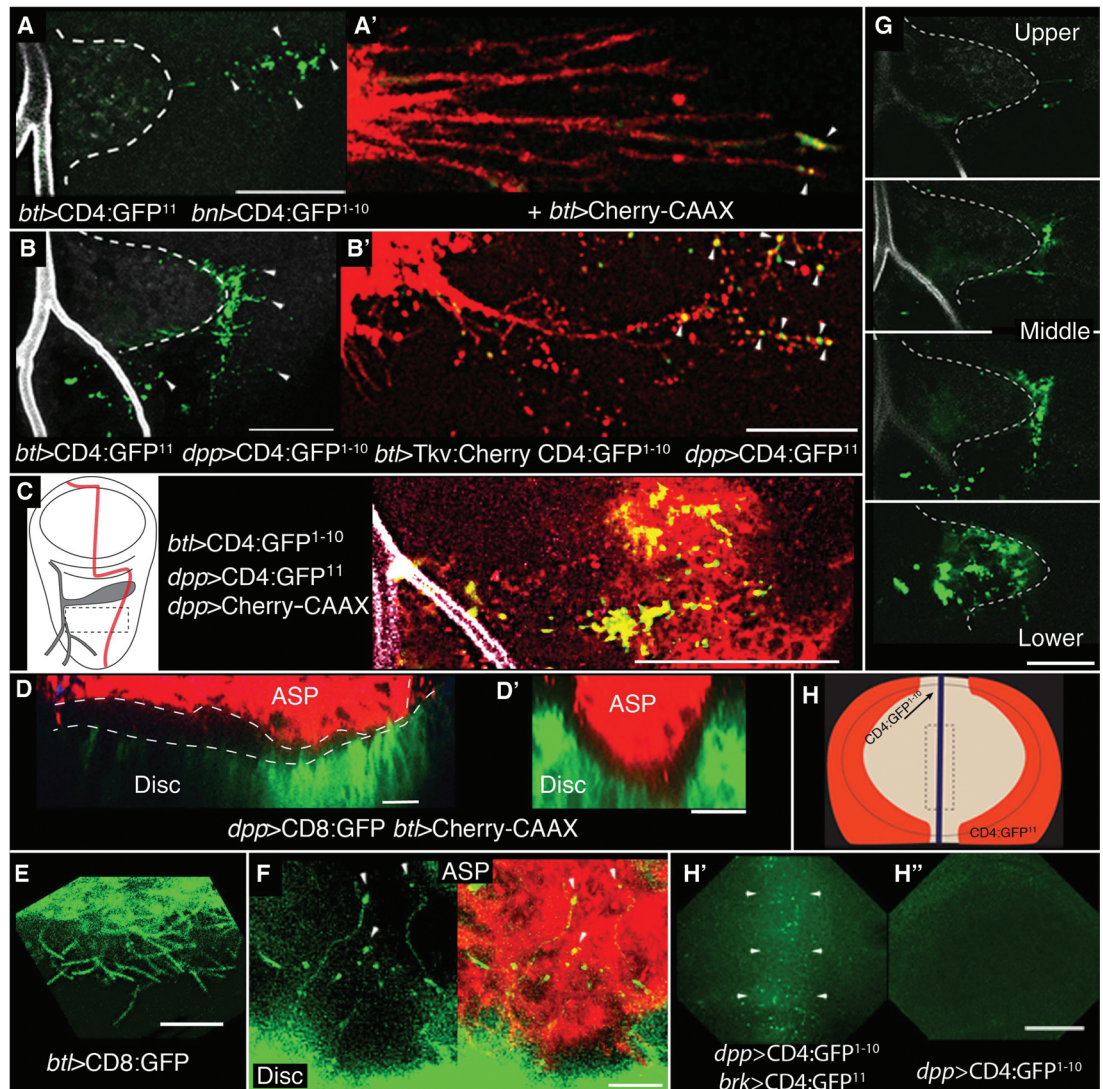
Lack of *caps* function also led to abnormal ASP development. Expression of *capsRNAi*, *trnRNAi*, or a dominant negative Caps mutant (Caps<sup>DN</sup>) that localizes similarly to wild-type Caps in synapses and decreases synaptic contacts (30, 32) reduced Dpp signaling and yielded abnormal ASPs (14) (Fig. 6B; fig. S1, L to N; and tables S2 and S3). Phenotypes were more extreme in a heterozygous *caps trn* double-mutant background. Expression of Caps<sup>DN</sup> did not cause detectable changes to cell polarity, cell morphology, mitotic activity, or cell viability (fig. S2, G and H).

Caps<sup>DN</sup> reduced amounts of dpERK (Fig. 6C), indicating that *caps* function was also required for FGF signaling. Evidence that signal transduction per se was not abrogated in ASP cells that lack *caps* function was obtained by overexpressing FGF ubiquitously. Heat shock–induced expression of FGF or expression of Dpp:Cherry in the ASP increased amounts of dpERK or pMad, respectively, throughout the ASP, attenuating the effects of Caps<sup>DN</sup> (fig. S3D). These experiments show that Dpp and FGF proteins that are produced by the disc (Fig. 1) (9) require *caps* function in the ASP to activate signal transduction in ASP cells, and show that mutant ASP cells that cannot receive FGF and Dpp from the disc are competent for signal transduction.

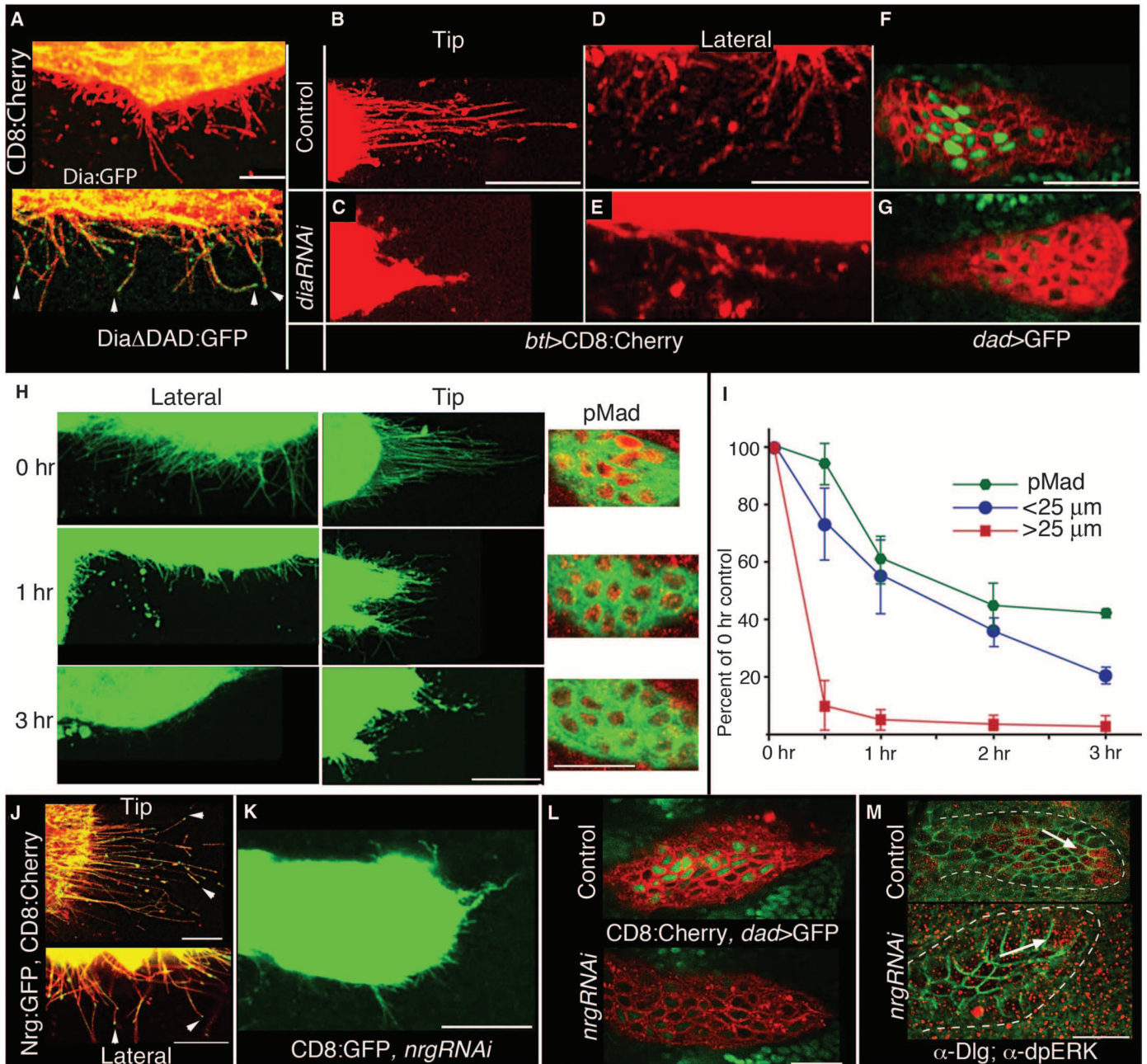
The presence of Caps:GFP in the tips of cytonemes (Fig. 6A), the role of Caps at neuronal synapses (30), the fact that cytonemes make contact with Dpp-producing cells (Fig. 4,

B, C, and G) and receive Dpp at apparent points of contact (Fig. 3, E and F), and the essential role of *caps* for Dpp signaling suggest that Caps may be required for cytonemes to establish functional contacts for Dpp exchange. However, the number and distribution of ASP cytonemes did not change under *caps* loss-of-function conditions (fig. S2I), indicating that the ASP cells do not require Caps to make cytonemes. In contrast, the contacts that ASP cytonemes made with Dpp-expressing disc cells required *caps*. We monitored these contacts with GRASP fluorescence: GFP fluorescence at the interface of Dpp-expressing disc cells and the lower layer of the ASP, and at cytoneme contacts of the lateral ASP and TC was reduced when Caps<sup>DN</sup> was expressed in the trachea (Figs. 1D; 4, C and D; and 6, D and E). In addition, Caps<sup>DN</sup> reduced uptake of Dpp:GFP from the disc (Fig. 6F), suggesting that although ASP cells make cytonemes in the absence of

**Fig. 4. Tracheal cytonemes contact Dpp- and FGF-expressing disc cells.** (A, A', B, and B') Green fluorescence (arrowheads) from reconstituted GFP (GRASP) due to contact between ASP cytonemes and disc shown in projection images composed of several "upper" to mid optical sections. ASP (dashed white line), disc, and TC lumen were imaged at 405 nm for background fluorescence (gray). Normal *dpp* expression includes cells anterior to the stripe at the A/P compartment border (see Fig. 1, A and B). Marking cytonemes with Cherry-CAAX (A') or Tkv:Cherry (B') showed that GRASP fluorescence was cytoneme-associated (arrowheads). (C) Left panel: drawing of third instar wing disc depicting Dpp-expressing cells (red) and ASP and TC (gray). Right panel: region outlined by dashed lines in left panel for GRASP fluorescence (green) at the basal surface of *dpp*-expressing disc cells (red). (D and D') Sagittal (D) and transverse (D') sections in the mid-region of ASP show the spatial relationship of the ASP (red) lower layer to *dpp*-expressing disc cells (green, *dpp*-CD8:GFP; red, *btI*-Cherry-CAAX). (E) CD8:GFP expressed in the ASP marks cytonemes emanating from the lower aspect of the ASP; they orient toward the disc. (F) CD8:GFP expressed in the disc marks cytonemes that extend toward and appear to contact (arrowheads) ASP cells marked with Cherry:CAAX (*btI*-Cherry:CAAX *dpp*-CD8:GFP). (G) GFP reconstitution in four optical sections of (B) from the upper layer, from the two middle layers, and from the interface between lower layer and disc. (H) Drawing of the wing pouch region of a wing disc showing the stripe of *dpp* expression at the organizer (purple) and the flanking regions that express *brinker* (*brk*,



orange). Box with dashed line indicates region imaged in (H') and (H''). (H') Reconstituted GFP (arrowheads) in the organizer region in disc with expression of the GFP fragments in the *brk* and *dpp* domains. (H'') Control with CD4:GFP<sup>1-10</sup> expression in the *dpp* domain only. Scale bars, 30  $\mu$ m, except for (A), (B'), (E), and (F), 10  $\mu$ m.



**Fig. 5. ASP cytonemes require *dia* and *Shi* and *nrg*.** (A) In the ASP, localization of Dia:GFP is predominantly in the cell bodies; activated Dia (Dia $\Delta$ Dad:GFP) localizes to cytoneme tips (*btl*-Gal4, *UAS*-CD8:Cherry/+; *tub*-Gal80<sup>ts</sup>/*UAS*-Dia:GFP, or *UAS*-Dia $\Delta$ Dad:GFP). (B to G) Expression of *diaRNAi* shortened lateral and tip of ASP cytonemes (*btl*-Gal4, *UAS*-CD8:Cherry/*tub*-Gal80<sup>ts</sup>; *Dad*-GFP/*UAS*-*diaRNAi*), and reduced expression of *Dad*-GFP. Control genotype: *btl*-Gal4, *UAS*-CD8:Cherry/*tub*-Gal80<sup>ts</sup>; *Dad*-GFP/+. (H and I) Late third instar larvae that coexpressed *sh<sup>ts1</sup>* and CD8:GFP (*btl*-Gal4, *UAS*-CD8:GFP, *UAS*-*sh<sup>ts1</sup>*) were incubated at 30°C for the indicated times and after dissection; GFP fluorescence and  $\alpha$ -pMad staining (red) were imaged in the lower layer of the ASPs (see Fig. 1C). The perimeter of each of the five ASPs was measured, cytonemes were counted (I) around the perimeter in about 35 to 40 optical sections, and the length of each cytoneme was measured. Graph (I) shows the average percentage change to the number of ASP cytonemes per micrometer perimeter in the length ranges of <25  $\mu$ m (blue) and >25  $\mu$ m (red). Amounts of pMad were determined by measuring

the mean fluorescence intensity (555 nm) in four ASPs for each time point for a region of the lower ASP level that contained about 11 cells. (J) Nrg:GFP (*btl*-Gal4, *UAS*-CD8:Cherry/*UAS*-Nrg:GFP; *tub*-Gal80<sup>ts</sup>) localizes to and concentrates at the tips (arrowheads) of ASP cytonemes. (K) Late third instar larvae that coexpressed *nrgRNAi* and CD8:GFP (*btl*-Gal4, *UAS*-CD8:GFP/*UAS*-*nrgRNAi*; *tub*-Gal80<sup>ts</sup>/+) . Lateral and tip cytonemes were stunted and reduced in number. (L) Expression of *Dad*-GFP was reduced in a lower ASP layer that expresses *nrgRNAi* (lower panel; *btl*-Gal4, *UAS*-CD8:Cherry/*UAS*-*nrgRNAi*; *Dad*-GFP/*tub*-Gal80<sup>ts</sup>) compared to control (upper panel; *btl*-Gal4, *UAS*-CD8:Cherry/+; *Dad*-GFP/*tub*-Gal80<sup>ts</sup>). (M) dpERK staining (arrows, red) is partially reduced in ASP that expresses *nrgRNAi* (lower panel, *btl*-Gal4/*UAS*-*nrgRNAi*; *tub*-Gal80<sup>ts</sup>/+; upper panel, control *btl*-Gal4/*UAS*-*nrgRNAi*; *tub*-Gal80<sup>ts</sup>/+); outline of ASP marked with dashed line and  $\alpha$ -Dlg (green) outlines cells. Conditions for conditional inactivation are described in table S2 (14). Scale bars, 25  $\mu$ m.

Caps function, Caps-deficient cytonemes that do not make stable synapses do not transfer Dpp from producing to recipient cells.

## Discussion

This study revealed an essential role for cytoneme-based transport of signaling proteins in long-distance paracrine signaling. This mechanism involves contact-dependent transfer of signaling proteins from producing to responding cells, and although we studied signaling between two epithelial tissues in a *Drosophila* larva, evidence from other systems supports a general role for cytonemes in paracrine signaling.

Studies of cells in culture indicate that filopodia receive and transport signaling proteins that are taken up from culture medium. In experiments with human adenocarcinoma cells, uptake of epidermal growth factor (EGF) protein from the culture medium led to retrograde transport by filopodia along with activated EGF receptor (EGFR) and was sensitive to cytochalasin D, a disruptor of F-actin (33). Actin-based cytonemes

that carry FGFR-rich puncta and that are dependent on the small GTPase (guanosine triphosphatase) RhoD are present in cultured mouse mesenchymal cells (34).

Some characteristics of Dpp signaling in the ASP are consistent with these cell culture experiments. Dpp that was taken up by an ASP cell was present in motile puncta that translocated along the ASP cell's cytoneme, and some puncta in the ASP cytonemes contained both Dpp and its receptor (Figs. 2, C and D; 3, C, E, and F; and 4B'). *Drosophila* cytonemes are actin-based (3). However, in contrast to cultured cells, signaling in the ASP did not appear to involve uptake of signaling proteins from the extracellular milieu, but was dependent on synaptic contact between the tip of a cytoneme that extended from a responding ASP cell and the cell body of a Dpp-expressing disc cell. This signaling mechanism appears to involve specific dynamic interactions between signaling and responding cells.

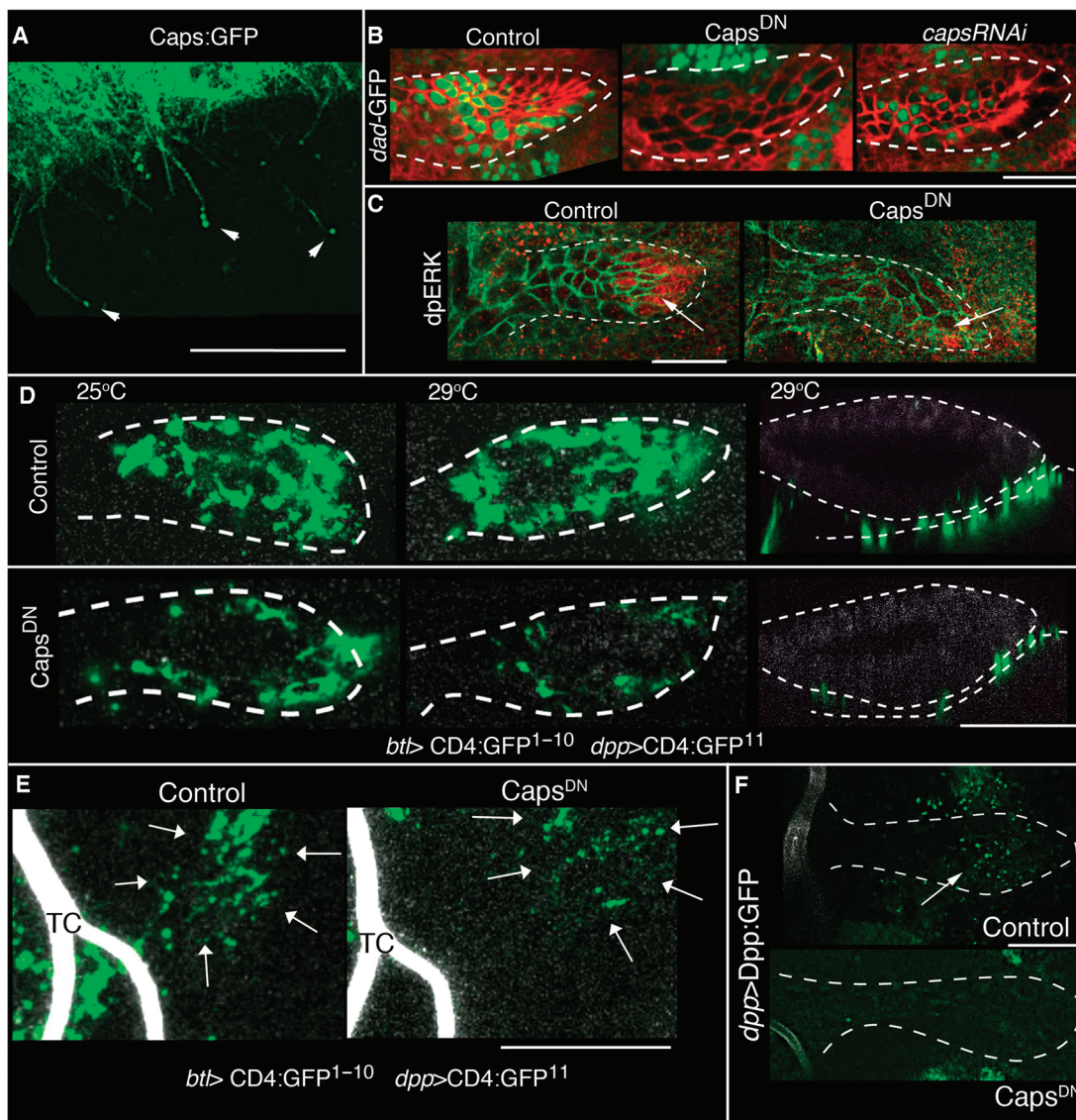
ASP cells express both the Tkv Dpp receptor and FGFR, and segregate these receptors to

puncta in distinct cytonemes (5). At the early L3 stage, the ASP is small and does not extend across the disc, and both the Dpp- and FGF-expressing disc cells are distal to its tip. Both Tkv- and FGFR-containing cytonemes extended distally from the tip (5). The FGFR-containing cytonemes extended beyond the Dpp-expressing cells and did not take up Dpp (Fig. 3D). At later L3 stages, the ASP has grown across the disc, and although the FGF-expressing disc cells are distal to it, the Dpp-expressing disc cells lie under its medial region. In these ASPs, the Tkv-containing cytonemes emanated from the medial region and reached as much as 40  $\mu\text{m}$  to pick up Dpp from disc cells (Fig. 3, B and C). Thus, in the ASP, spatially restricted Dpp signal transduction (Fig. 1, C and F) and uptake (Figs. 2A and 3, B and C) were associated with cytonemes whose orientation and composition appeared to be specific for Dpp.

The dynamism of this signaling system may be inferred from steady-state images. The distribution and orientation of cytonemes change if expression of signaling protein is compromised

### Fig. 6. ASP cytonemes require Caps.

**(A)** Caps:GFP (*btl-Gal4 UAS-Caps:GFP, tub-Gal80<sup>TS</sup>*) localizes to and concentrates at the tips (arrowheads) of cytonemes. Conditions for conditional inactivation are described in (14). **(B)** ASP expression (*btl-Gal4*) of Caps<sup>DN</sup> (middle panel) and *capsRNAi* (right panel) reduced *Dad* expression in the ASP (*Dad-nlsGFP*; green); left panel, control. **(C)** ASP expression (*btl-Gal4*) of Caps<sup>DN</sup> reduced dpERK staining (red) at the tip of ASP. Cells are marked with  $\alpha$ -Dlg staining [red, (B); green, (C)]. **(D)** Sagittal optical sections at lower level of ASP (left and middle panels) and in coronal sections (right panels) showing that GRASP fluorescence is reduced by expression of Caps<sup>DN</sup> (at 29°C); Caps<sup>DN</sup> genotype includes two copies of *UAS-Caps<sup>DN</sup>*. TC indicates the lumen of the transverse connective. **(E)** Caps<sup>DN</sup> expression in the TC reduces GRASP fluorescence (arrows) associated with Dpp-expressing disc cells. Genotypes: same as (D). **(F)** Dpp:GFP uptake in ASP (arrow) in the presence (bottom panel) and absence (top panel) of Caps<sup>DN</sup>. Genotypes: same as (D). In (D) to (F), ASP, disc, and TC are imaged for autofluorescence at 405 nm (gray). Scale bars, 30  $\mu\text{m}$ , except for (A), 10  $\mu\text{m}$ .





and if signaling protein is overexpressed in ectopic locations (4, 5, 9). These properties suggest that cytonemes are changeable and that their distributions reflect the relative positions of signal-producing and signal-receiving cells. The different distributions of Tkv-containing cytonemes in the temporal sequence described above are consistent with this idea and with a model of cytoneme impermanence. The observation that some ASP cytonemes contain Tkv, make contact with Dpp-producing cells, and take up Dpp, whereas other cytonemes contain Tkv but do not make contact with Dpp-producing cells or take up Dpp (Figs. 2E and 3F), may also suggest that cytoneme contacts may be transient.

Plasticity may be an important attribute of cytonemes that function in a developmental system such as the ASP, in which relations between producing and receiving cells change as the larva develops. Cytonemes may have the capacity to regulate release and uptake of signals and to direct signals to a preselected target. Regulated release may be implied by the absence of Dpp uptake and Dpp signal transduction in ASP mutant conditions that abolish synaptic contacts by ASP cytonemes. In these experiments, the signal-producing cells were not mutant, and the wing discs, which depend on Dpp signaling, developed normally, indicating that the signaling defect was specific to the ASP cells that made defective cytonemes. Because filopodia of cultured cells take up signaling proteins from culture medium and activate signal transduction (33), we may assume that ASP cytonemes are similarly capable of taking up signaling protein that their receptors encounter and that the inability of cytoneme-defective cells to take up Dpp or activate signal transduction may indicate that Dpp was not released in the absence of cytoneme contact.

There may be a functional analogy to neuronal signaling. Neurons make asymmetric extensions that send and receive signals, make specific contacts where signal release and uptake are regulated, and require the *diaphanous*, *neuroglian*, *shibire*, and *capricious* genes for contact-mediated signal exchange and signaling. In the developing *Drosophila* retina, Hh moves to the termini of retinal axons, where it signals to postsynaptic laminal neurons in the brain (35). Perhaps the strongest precedent is Wingless delivery at developing neuromuscular junctions in the *Drosophila* larva; in this case, Wingless moves to the postsynaptic cell after release in a vesicular form from the presynaptic neuron (36). Our studies have been limited to the cytonemes that are made by receiving cells, but in other contexts, cytonemes extend from cells that deliver signaling proteins, such as the Hh-containing cytonemes of the wing disc (7) and the cytonemes that extend from cap cells in the female germline stem cell niche (8) and that are associated with Notch and EGF signaling (37–40). Cytonemes that transport Hh across the chick limb bud from Hh-producing cells have also been described (41). The widespread presence of cytonemes in many cell types

and in many contexts suggests that they may provide a general mechanism to move signaling proteins between nonneuronal cells at sites of direct contact.

## Materials and Methods

### Drosophila Stocks

Transgenes: *btl-Gal4* (9), *ap-Gal4* [Bloomington Stock Center (BSC)]; *dpp-Gal4/CyO*; *HS-Bnl* (9); *UAS-Tkv:GFP* (4); *UAS-Dpp:Cherry*, *UAS-CD8:Cherry*, *UAS-CD8:GFP* (5); *Dad-nEGFP* (III) (42), *UAS-FGFR<sup>DN</sup>* (43); *dpp-LHG/TM6* (LexA-Gal4 activation domain fusion; III) (44), *dpp-LHG* (II; this study), *lexO-Dpp:GFP/TM6* and *brk<sup>BM14</sup>-LHG* (44), *btl-LHG* (II and III) (this study), *lexO-CD4:GFP<sup>11</sup>* (II), *UAS-CD4:GFP<sup>1-10</sup>* (III) (17), *UAS-dppRNAi* (BSC), *UAS-putRNAi* (BSC); *tub-Gal80<sup>85</sup>* (II and III; BSC), *UAS-Dad* (II) (BSC), *UAS-Tkv<sup>OD</sup>/TM6B* [activated Tkv (45)], *UAS-Tkv<sup>DN</sup>*, *UAS-Put<sup>DN</sup>* [ $\Delta$ GSK, dominant negative forms of Tkv and Put lacking GS and kinase domain (46)], *UAS-TIPF* (15), *UAS-capsRNAi* [BSC, Vienna Drosophila RNAi Center (VDR)], *UAS-trnRNAi* [National Institute of Genetics (NIG), BSC], *UAS-Caps<sup>DN</sup>* (30); *UAS-CD4:GFP<sup>10</sup>* (II; this study), *UAS-diaRNAi* (BSC, NIG), *UAS-shi<sup>ts1</sup>* (24), *lexO-CherryCAAX* [II and III (44)]; *UAS-Dia:GFP* (47) and *UAS-DiaDad:GFP* (23); *UAS-mrgRNAi* (II and III) (BSC); *10XUAS-IVS-mCD8::RFP*; *13XlexO-mCD8::GFP* (BSC); *UAS-Nrg:GFP* (II) (27).

Insertions and mutations: *Dad<sup>11E4</sup>-LacZ/TM3*, *tkv<sup>K16713</sup>-LacZ/CyO*, *dpp<sup>10638</sup>-LacZ/CyO*, *put<sup>10460</sup>-LacZ/TM3* (BSC), and *Nrg:GFP* protein trap line (flytrap line G00305). Conditional inactivation of Dpp was in *dpp<sup>4</sup>/dpp<sup>56</sup>* L3 larvae for 18 hours at 29°C as described (4).

### Overexpression

*tub-Gal80<sup>85</sup>* was present to limit expression to the L3 stage. Expression drivers were as follows: *ap-Gal4* for *dppRNAi*; *btl-Gal4* for *Dad*, *Tkv<sup>DN</sup>*, *Tkv<sup>OD</sup>*, *Put<sup>DN</sup>*, *putRNAi*, *Caps<sup>DN</sup>*, *capsRNAi*, *trnRNAi*, *diaRNAi*, *Dia:GFP*, *DiaDad:GFP*, *mrgRNAi*, and *Nrg:GFP*. Animals were reared at 18°C until L3 and were incubated at 29°C, as indicated in table S2, before dissection. For knock-down under heterozygous mutant background (table S2 and fig. S1M), *Caps<sup>DN</sup>* and *capsRNAi* expression was driven by *btl-Gal4* or by *dpp>Gal4* in *caps<sup>C29fs</sup> trn<sup>Δ17</sup>* and *caps<sup>65.2</sup> trn<sup>S064117</sup>* double mutants. At 25°C, *Caps<sup>DN</sup>* and *capsRNAi* overexpression is embryonic lethal in the *Caps* mutant background; animals were therefore reared at 20°C to the L3 stage and were incubated at 25°C for 1 day before dissection.

### Ectopic Expression

For fig. S3, A to C, crosses were, for *dia*, *shi*, and *mrg*: *btl-Gal4*, *UAS-CD8:GFP/+*; *tub-Gal80<sup>85</sup>/UAS-dpp:Cherry* to either *UAS-diaRNAi*, *UAS-shi<sup>ts1</sup>*, or *UAS-mrgRNAi*. Control larvae expressed either *shi<sup>ts1</sup>*, *diaRNAi*, or *mrgRNAi*, but lacked *dpp:Cherry*; experimental larvae had *UAS-dpp:Cherry*. Ani-

mals were reared at 18°C to minimize the effects of Dpp overexpression. To express *diaRNAi*, L3 larvae were incubated at 25°C for 5 to 6 hours. *Shi<sup>ts1</sup>* larvae were treated similarly and were then shifted to 29°C for 1 hour. ASPs in the *Shi<sup>ts1</sup>* larvae did not grow normally because of temperature sensitivity of *shi<sup>ts1</sup>* at 25°C. *mrgRNAi* induction was for 14 to 18 hours at 29°C. *Caps<sup>DN</sup>* larvae (*btl-Gal4*, *UAS-CD8:GFP/UAS-Caps<sup>DN</sup>*, *UAS-Caps<sup>DN</sup>/HS-Bnl*) were reared at 20°C until L3; heat shock induction of Bnl was for 30 min at 37°C followed by 3 hours of incubation at 20°C.

### Dual Expression

LexA and Gal4: *10XUAS-IVS-mCD8::RFP*, *13XlexO-mCD8::GFP* flies (BSC) were crossed to *dpp-Gal4/SM5*; *btl-LHG* flies to mark Dpp-producing cells in wing disc with RFP (red fluorescent protein) and trachea with GFP. To express either Tkv:Cherry or FGFR:Cherry in trachea simultaneously with Dpp:GFP in the wing disc, *UAS-Tkv:Cherry/CyO-Weep*; *dpp-LHG/TM6* or *UAS-FGFR:Cherry/CyO-Weep*; *dpp-LHG* flies were crossed to *btl-Gal4*; *lexO-Dpp:GFP/TM6* flies. To minimize toxic effects, *btl-Gal4/UAS-Tkv:Cherry* (or *FGFR:Cherry*); *lexO-Dpp:GFP/dpp-LHG* animals were grown at 18°C until the L2 stage and were shifted to 20°C.

### Enhancer Trap Screening

About 500 lines with randomly inserted enhancer trap transposons (gift from E. Heberlein) were screened for tracheal expression (*UAS-GFP*). A line with elevated expression in the ASP was identified; its Gal4 transposon was mapped by ends out sequencing to the first exon of *caps*. Wing disc GFP expression was similar to the expression of *caps* as indicated by in situ hybridization (28).

### GFP Reconstitution

Genotype for reconstitution between Dpp signaling partners: *dpp-Gal4/lexO-CD4:GFP<sup>11</sup>*; *btl-LHG/UAS-CD4:GFP<sup>1-10</sup>*. Genotype for reconstitution between FGF signaling partners: *btl-LHG/lexO-CD4:GFP<sup>11</sup>*; *btl-Gal4/UAS-CD4:GFP<sup>1-10</sup>*. For reconstitution in the wing disc: *dpp-Gal4/lexO-CD4:GFP<sup>11</sup>*; *brk-LexA/UAS-CD4:GFP<sup>1-10</sup>*. For reconstitution with marked cytonemes: *btl-LHG, lexO-CherryCAAX/lexO-CD4:GFP<sup>11</sup>*; *btl-Gal4, btl-LHG/UAS-CD4:GFP<sup>10</sup>*. For reconstitution with in the presence of marked Tkv: *btl-Gal4, dpp-LHG/UAS-Tkv:Cherry; lexO-CD4:GFP<sup>11</sup>*, *UAS-CD4:GFP<sup>1-10</sup>*. For reconstitution with marked Dpp source: *btl-Gal4, dpp-LHG/+; lexO-Cherry:CAAX/UAS-CD4:GFP<sup>1-10</sup>*, *lexO-CD4:GFP<sup>11</sup>*. For reconstitution together with *Caps<sup>DN</sup>* overexpression, *btl-Gal4, dpp-lexA; UAS-CD4:GFP<sup>1-10</sup>*, *lexO-CD4:GFP<sup>11</sup>* flies were crossed with either *w* (control) or *UAS-Caps<sup>DN</sup>*. Larvae were reared in room temperature and shifted to 25° or 29°C for 1 day before assay.

### shibire Inactivation

Larvae [*btl>Gal4*, *UAS>CD8:GFP*, *UAS>shi<sup>ts1</sup>* (24)] were raised at 18°C before a single heat shock for 0.5, 1, 2, or 3 hours at 30°C. Larvae were dis-

sected and imaged for ASP cytonemes or were fixed for pMad staining. Rescue after heat shock was by returning larvae to 18°C before dissection and imaging. Control heat shock was with larvae expressing CD8:GFP in trachea (*btl-Gal4 UAS-CD8:GFP*) at 30°C for 0 and 3 hours. No significant change in numbers of cytonemes [either <25 μm (4.4 ± 4.7% reduction) or >25 μm (4.7 ± 7.6% increase)] was detected. Rescue after 30°C at 2 hours was at 20°C for 1 hour, followed by dissection and imaging. Increases in numbers of cytonemes [<25 μm (1.9 × ± 0.4%, *P* = 0.0471) or >25 μm (11 × ± 2.9%, *P* = 0.0196)] were evaluated by the unpaired *t* test.

### Quantitation and Statistical Analyses

Cytonemes were counted and measured in z-section stacks of confocal images from five ASPs for each data point and were binned as <25 or >25 μm. Lengths represent measures from each tip along the connecting shaft to the point of its widening base either at the plasma membrane or at the lamellipodia-like protrusion. The size variation between ASPs was normalized by measuring the perimeter of each ASP and then calculating the number of cytonemes per unit length. Values in Fig. 5I are plotted as percentage of the 0-hour time point. pMad levels were quantified by measuring the mean intensity of 555-nm fluorescence in the cells of the lower layer of ASP, subtracting background fluorescence, and normalizing with respect to pMad fluorescence at the A/P border of the same wing disc. Values were plotted as percentage of the 0-hour time point. Statistical significance values were calculated with *t* test or analysis of variance (ANOVA) followed by Tukey honestly significant difference (HSD) test.

### Molecular Cloning

*btl>LHG*: The P[B123] fragment upstream of the *btl* gene (48) was amplified from a genomic clone obtained from (49), with 5' primer GGCTCGA-GATAATCGCATTCCTGACCTCGGTAAAC and 3' primer GGTCTAGAGGATCGTACCCGTAATCCG, and the product was cloned in *pCASPER4*. The LexA:Gal4H-GAD portion was isolated from the *pDppattB-LHG* plasmid (44) and was inserted at the *pCASPER4* Not I site.

*Tkv:Cherry*: The Not I-Hind III fragment from a *Tkv:GFP* construct (4) was ligated to a mCherry fragment with 5' Hind III and 3' Kpn I sites in the presence of *pUAST* that had been digested with Not I and Kpn I. Primers for mCherry amplification: 5' primer, GCAAGCTTATGGTGGCAAGGGC-GAGGAGG; 3' primer, AGGTACCTTACTTG-TACAGCTCGTCCATGCCGC. *Tkv:Cherry* and *Tkv:GFP* are similar in phenotype, activity, and localization in cytonemes.

### In Situ Hybridization

RNA in situ hybridization was performed according to (50). Digoxigenin (DIG)-labeled antisense probe was generated by transcription from a T7 promoter joined to a 600-base pair fragment of *dpp* complementary DNA (cDNA) amplified with

polymerase chain reaction primers: CAAGGAGGC-GCTCATCAAG and TTGTAATACGACTCACT-ATAGGGAGACACCAGCAGTCCGTAGTTGC. Alkaline phosphatase-conjugated α-DIG antibody (Roche) was used to detect the DIG-labeled probe.

### Immunohistochemistry

The following antisera were used: α-pMad [from E. Laufer and P. ten Dijke; at 1:2000 (51)]; α-dpERK (Sigma; 1:250) and α-apontic [from R. Schuh (52)]; and α-discs large (4F3; 1:50), α-DE-cadherin (DECAD2; 1:20), and anti-β-galactosidase (Developmental Studies Hybridoma Bank). dpERK staining was carried out as described (9) with antibody obtained from Cell Signaling Technology. Secondary antibodies were conjugated to Alexa Fluor 488, 555, or 647. To assay for cell lethality, α-cleaved caspase-3 (Asp<sup>175</sup>; Cell Signaling Technology) was used as described (53). Cell proliferation was monitored with α-phosphohistone H3 antibody (Ser<sup>10</sup>; Cell Signaling Technology).

### Imaging Techniques

Wing discs were dissected and mounted as described (5), except that the second small coverslip was omitted. Images were made with a Leica TCS SPE or TCS SP2 confocal microscope with either 405, 488, 561, or 635 wavelength lasers and with LAS-AF software; or with a custom-built Zeiss spinning disc confocal with electron-multiplying charged-coupled device (EM-CCD) Hamamatsu camera (9100-13) and Velocity 5.5 software; or with a standard Zeiss AxioPlan 2 fluorescence microscope with sensicam CCD camera (Cooke Corporation) and SlideBook 4 acquisition software (Intelligent Imaging Innovations). Patterns of cytonemes were consistent in all three types of systems. Brightfield images were made on a Leica DMR microscope equipped with SPOT CCD camera (Diagnostics Instruments) and SPOT acquisition software. Final images were analyzed and processed with National Institutes of Health (NIH) ImageJ.

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#### Supplementary Materials

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