EXPERIMENTAL PROCEDURES

Drosophila stocks
FLP-out clones: *hsFlp; abxubx>FRT>GAL4, UAS-CD8:GFP/SM5*
FLOXed-out clones: HS-*Cre*PR*, *tubulin>LOXP>* and *tubulin>LOXP>dpp:cherry* (this study, see below)
Ectopic expression: *HS-bnl* and *btl-GAL4* (*1*); *HS-dpp* and *UAS-tkv:GFP* (*2*); *HS-hh, HS-cspi, UAS-btl:Cherry*, *UAS-dpp:cherry, UAS-cherry, UAS-CD8:Cherry* (this study); *UAS-EGFR:EGFP* (*3*); *UAS-EGFR* (*4*), *UAS-EGFR[DN]* (*5*), *UAS-CD8:GFP*, *dad-lacZ* (dad^{j1E4}*/TM3), tkv-lacZ* (tkv^{k16713}*/CyO), dpp-GAL4/CyO* (Bloomington Stock Center).

Molecular cloning All GFP constructs were with eGFP (*6*) either fused either to the transmembrane domain of mouse lymphocyte CD8 protein (*7*), EGFR or Tkv. pFlox vector: pCasper-4 (*7*) modified to have, in order: *tubulin* promoter - Flox - PTSV40 (SV40 poly(A), with STOP) – Rlox – Multiple Cloning Site (XbaI, BglII, SphI, NheI, XhoI) – SV40 poly(A). HS-hCrePR pCaSpeR-hs modified to express CreV336A:PR650-914. This Cre*-PR* fusion of human Cre has a V336A mutation to inactivate a pseudo-splicing site and has enhanced specificity and increased sensitivity to RU486 (*8*). The progesterone receptor domain (residues 650-914) was reported to provide more ligand dependent activity and reduced background. *HS-hh* and *HS-spi*: pCaSpeR-HS was modified to express Hh and cSpi (*9*), respectively. Dpp:Cherry fusion: construct is based on the GFP:Dpp fusion of Entchev et al. (*10*). mCherry was inserted C-terminal to the processing site to generate: (Dpp)DVSGGEGGG-GT-MV(mCherry)YL-GS-KGGR(Dpp). Btl:Cherry fusion: mCherry was inserted C-terminal to *btl* coding sequence to generate Btl-VDGQG-mCherry.

Evidence that the biological activity of the transgene constructs used in this study:
HS-hh and *HS-spi*: heat shock of *HS-hh* and *HS-Spi* flies yielded expected adult wing and eye phenotypes, respectively.
Dpp:Cherry: Expression of *UAS-Dpp:Cherry* driven by *dpp-GAL4* generated fluorescent apical/ sub-apical intracellular puncta distributed in a gradient toward the lateral flanks of the wing disc, and in a random distribution around somatic clones of expressing cells as described for similar Dpp:GFP fusion proteins (*10-12*). Expression of *UAS-Dpp:Cherry*, *UAS-Dpp:GFP* and *UAS-Dpp* driven by *vg-Gal4* generated phenotypes that were indistinguishable.
Btl:Cherry: Expression of *UAS-Btl:Cherry* driven by *btl-Gal4* was viable but could not be balanced in a stock; *btl-Gal4 UAS-Btl:Cherry HS-bnl UAS-CD8:GFP* was inviable at 18°C, but could survive in the presence of $Gal80^{ts}$. The presence of Btl:Cherry enhanced the severity and rapidity of response in ASP cells to induced expression of *HS-bnl* (Fig. 3 and Fig. S3).

Tkv:GFP: Clones expressing Tkv:GFP driven by *abxubx-Gal4* or *actin-Gal4* in wing disc were abnormally small in size and rare, indicating expected sensitivity to level of Tkv expression and confirming previous characterizations (*2, 13*). *btl-Gal4; UAS-Tkv:GFP*: Homozygous viable and normal phenotype. Increased expression of Tkv:GFP with two copies of *btl-Gal4* resulted in morphological phenotype in tracheal branches.

$EGFR^{DN}$ has an inactive kinase domain (*5*)

Generation of FLP-out clones Random FLP-out clones were induced in *HS-FLP; abxubx>FRT>GAL4,UAS-CD8:GFP/SM5* flies by incubating early 3rd instar larvae for 12 minutes in a 37°C water bath. Larvae were returned to 25°C and were sacrificed 36-48 hr later. GFP fluorescence marked the clonal descendents of the recombined parent; no phenotype was associated with expression of CD8:GFP.

Generation of FLP-out and FLOX-out clones Random FLP-out CD8:GFP clones were induced in *HS-FLP; abxubx>FRT>GAL4,UAS-CD8:GFP/SM5* flies by incubating early 3rd instar larvae for 12 minutes in a 37°C water bath. Random FLOXed-out clones were generated in *tubulin>LoxP>dpp:Cherry / + ; HS-Cre*PR* / +* flies with the same regimen of heat shock followed by treatment with RU486. To generate wing discs with clones that express both CD8:GFP or Dpp:Cherry, *abxubx>FRT>GAL4,UAS-CD8:GFP/SM5; HS-CrePR* and *HS-Flp; tubulin>LoxP>dpp:cherry* flies were crossed. Early L3 larvae were subjected to a twelve minute heat shock to induce FLP-out clones, incubated for 2 hr at 25°C, bathed in 3mg/ml RU486 for 2 min, and then placed in food containing 0.013 mg/ml RU486 to induce clones of Dpp:Cherry-expressing cells.

Over-expression of signaling proteins by heat shock Early 3rd instar larvae were subjected to 12 min heat shock to induce recombination (as above), and then to a prolonged heat shock (37°C) approximately 36-48 hrs later to induce ectopic expression of a transgene. The second heat shock was titrated to express levels that were sufficient to generate characteristic adult phenotypes; 1 hr for Hh, cSpi and Bnl; and 2X1 hr, separated by1/2 hr at 25°C, for Dpp. Clones were monitored after 1/2, 1, 2, 3, 6 and 12 hr post heat shock; changes to cytonemes from columnar clones in wing and eye discs were maximal 1/2-3 hr post-induction and 3-5 hr for the ASP.

To express Bnl ubiquitously in larvae that also express Btl:Cherry in trachea, a scheme that included $Gal80^{ts}$ was used. Animals with the genotype *btl-Gal4;UAS-CD8:GFP/HS-bnl; UAS-Btl:Cherry* are lethal at the L1 stage, even at 17°C. In contrast, *btl-Gal4;UAS-CD8:GFP/HS-Bnl* larvae are viable at 17°C without tracheal defects, and cytoneme distribution is normal. To examine the distribution of Btl:Cherry in cytonemes after Bnl over-expression, *tubP-$Gal80^{ts}$* (tubulin promoter driven temperature sensitive Gal80) was

added to the genotype to suppress Gal4 expression at the permissive temperature. At the appropriate L2-L3 stage, *btl-Gal4 UAS-CD8:GFP/HS-Bnl ; UAS-Btl:Cherry/Gal80^{ts}* larvae were incubated at 25°C to inactivate Gal80^{ts} to induce Gal4 driven expression of both CD8:GFP and Btl:Cherry in trachea. HS-Bnl was induced 6-12 hr later and images were taken every hour for eight hours. Because the morphology of the ASP and the distribution of cytonemes was abnormal at 3-5 hrs (Fig. S3), we monitored cytonemes 1-2 hrs post HS. In contrast to the *btl-Gal4 UAS-CD8:GFP HS-Bnl* genotype in which long (>30μm) cytonemes were present at the ASP tip 3-5 hrs after heat shock, no long cytonemes were present more than 1 hr after heat shock in *btl-Gal4 UAS-CD8:GFP/HS-Bnl ; UAS-Btl:Cherry/Gal80^{ts}* larvae.

Imaging Discs were dissected and mounted as described (*2*). Fluorescence levels in cytonemes were too low to detect directly, but were viewed in recorded images after renormalization and enhancement so that cytonemes could be perceived against the background of non-expressing cells. Images were refined by de-convolution (Slidebook v4.0); some were also processed with the Adobe Photoshop Sharpen Filter. Figure S4H is a 3D projection assembled from all collected z sections using ImageJ program. Images in Fig S4 L-N were obtained with a Leica TCS SP2 confocal microscope and were processed with Leica Application Suit Advanced Fluorescence Lite 2.3.5 build 5379 software.

Statistical analysis

ASP tip cytonemes were counted manually across a 100 μm arc centered at the tip, and were grouped by length (either <30 μm or >30 μm). This grouping is based on co-expression of Btl:Cherry and Tkv:GFP and the localization of Btl:Cherry to cytonemes 2/3 of which were >30 μm, and of Tkv:GFP to cytonemes that were all <30 μm (Table S5). P-values in Table S3 were calculated using unpaired t-tests for pair-wise comparisons of cytoneme counts pre- and post-heat shock between ASPs with the same genotype. A complementary analysis on the total number of cytonemes (both <30 μm and <30 μm) was performed by comparing the number of cytonemes from post-heat shocked HS-Hh, HS-Spi, HS-Dpp, and HS-Bnl larvae to post-heat shocked CD8:GFP larvae. Following the application of ANOVA, Dunnett's test was used to test for differences between each genotype and the CD8:GFP control.

References

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	% clones with cytonemes	Average length	Length range	Cell diameter	Maximum distance to signaling center
Wing blade columnar (apical) *	20	20.4	5-80.2	0.5-2.2	85 (P), 135(A)
Wing blade columnar (basal)	40	2	4-30	0.5-2.2	85 (P), 135(A)
Notum (apical) *	ND	7.4	1.8-12.7	0.5-2.2	NA
Eye disc, anterior	15	16	25	0.5-2.4	94-127
Eye disc, posterior	0	NA	NA	NA	50-81
Air sac primordium	NA	22.9	12-50	NA	NA
Transverse connective	NA	8.5	2-15	NA	NA
Myoblasts	95	14.5	12-28	variable	NA
Peripodial wing	30	18	5-38	15-22 (short)° 45-55 (long)	NA
Peripodial eye	45	25.5	6.4-42	7-15 (short) 10-20 (long)	NA

Table S1. Frequency and length distribution of apical cytonemes from various cells of wing and eye discs. Lengths are in μm. All measurements were from mid L3 larval stage. NA - not applicable; ND – not determined; *Combined data from this study and Hsiung et al, 2005; °short and long axes of oval shaped cells.

	clones		clones with cytonemes		wing disc cytonemes				eye disc cytonemes				ASP with cytonemes	
	wing disc	eye disc	wing disc	eye disc	to A/P		non-A/P		to MF		non-MF		to FGF source	other
					#	L	#	L	#	L	#	L		
HS-hh	65	53	8	6	8	5-15	0		5	5-12	1	4	30	0
HS-dpp	60	55	11	5	2	4-6	9	5-14	6	5-13	0		35	0
HS-bnl	58	59	6	5	6	4-13	0		5	5-19	0		3	33
HS-cspi	52	63	7	7	6	5-19	2	4-5	1	4	6	3-6	30	0
HS control	25	51	3	4	3	13-20	0		4	11-17	0		15	0

Table S2. Orientation of cytonemes after heat shock induction of Hh, Dpp, Bnl/FGF and cSpi/EGF

"L": length of the cytonemes in μm.

	CD8:GFP	HS-Hh		HS-Spi		HS-Dpp		HS-Bnl	
cytonemes	post-HS	pre-HS	post-HS	pre-HS	post-HS	pre-HS	post-HS	pre-HS	post-HS
<30 μm	15.2±1.9	20±5.6	25.6±4.3 *P=0.17*	21±3.8	24.4±6.5 *P= 0.35*	20.3±8.9	35±6.2 *P=0.0017*	19.8±5.8	51.6±28 *P=0.007*
>30 μm	7.4±2.3	6.8±1.7	4.2±2.5 *P=0.095*	6±1.6	5±1.4 *P= 0.32*	7.3±2.8	5.5±1.9 *P= 0.16*	10.8±2	35±17.6 *P=0.0017*

Table S3. Number and lengths of cytonemes at the ASP tip. Numbers represent averages numbers of cytonemes in a 100 μm circumferential arc centered at the ASP tip from 5-8 preparations. *P* values calculated by unpaired t-tests are for pair-wise comparisons between the total number of cytonemes <30 μm or >30 μm made for animals of the same genotype pre- and post- heat shock. The data for CD8:GFP, HS-Hh, HS-Spi, HS-Dpp and HS-Bnl post heat shock were analyzed by applying ANOVA, and the Dunnett's test was used to evaluate the changes in numbers of cytonemes between each genotype and the CD8:GFP control. This complementary analysis confirmed that the increased number of cytonemes after Bnl over-expression was significant ($P<0.0001$) as compared to the CD8:GFP control; the increased number of <30 μm cytonemes after Dpp over-expression as compared to the CD8:GFP control was not statistically significant ($P=0.09$).

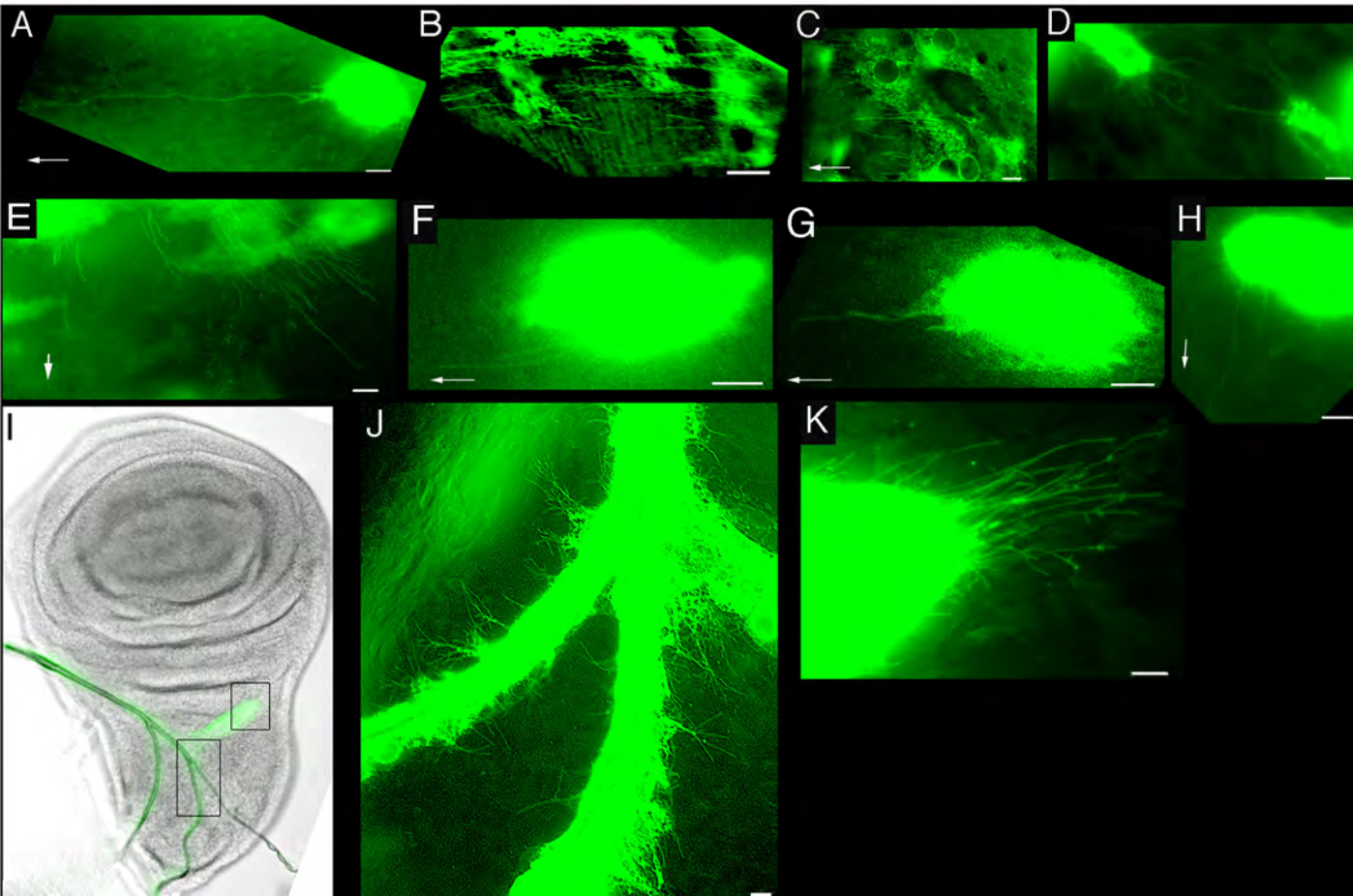
		Fraction of cytonemes with Btl:Cherry puncta								
	length (μm)	#1	#2	#3	#4	#5	#6	#7	#8	Fraction (total)
HS Control	>30	1/2	1/1	1/1	1/1	1/1	2/2	1/1	2/2	0.91
	<30	0/16	0/15	1/15	0/14	1/15	2/15	2/17	0/14	0.06
HS-Bnl Btl:Cherry	>30	4/4	2/2	3/3	2/2	0/0	0/0	0/0	0/0	1.0
	<30	16/47	20/40	8/29	9/25	20/25	11/22	17/50	11/23	0.43
HS-Dpp Btl:Cherry	>30	2/2	2/2	1/2	2/2	1/1	2/2	1/1	2/2	0.93
	<30	1/32	1/31	1/31	1/25	2/29	0/35	1/32	2/34	0.04

Table S4: Distribution of Btl:Cherry puncta in cytonemes at ASP tip. The total number of cytonemes as well as the number of cytonemes that contain Btl:Cherry puncta were counted within a 100 μm circumferential arc at the ASP tip in all z optical sections taken for eight preparations, of which preparation numbers 1-5 were made between 1-2 hrs after heat shock induction and numbers 6-8 were made at 2 hrs after heat shock induction. Fraction = # <30 μm cytonemes with puncta / total # <30 μm cytonemes; or # >30 μm cytonemes with puncta / total # >30 μm cytonemes.

	Cytoneme length				
Cytonemes with puncta	<20μm	20-30μm	30-40μm	40-50μm	50-60μm
Btl:Cherry-containing	2	4	7	5	6
Tkv:GFP-containing	131	30	0	0	0

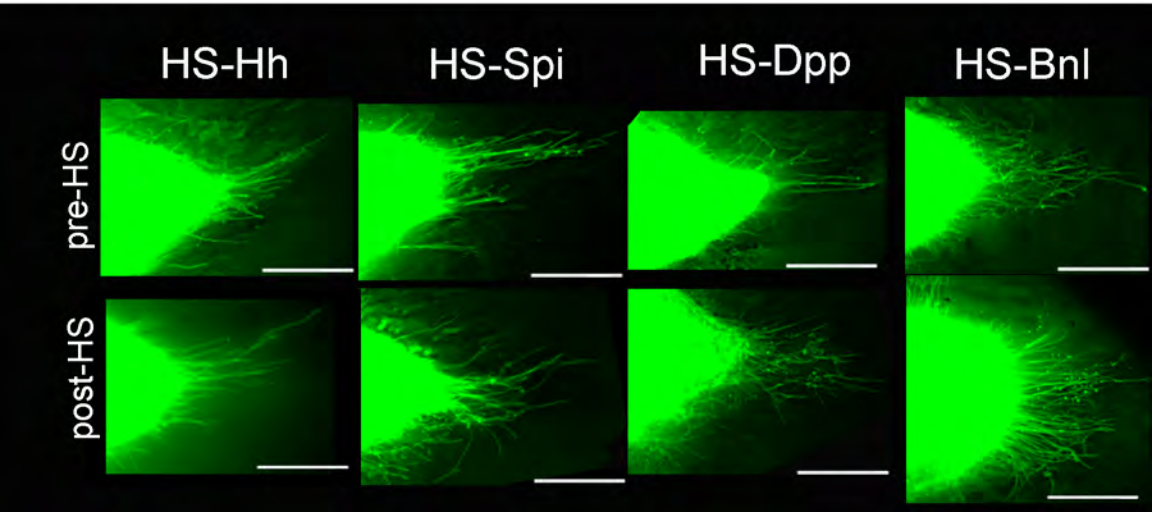
Table S5. Lengths of cytonemes containing Btl:Cherry or Tkv:GFP Total numbers of cytonemes of indicated lengths in fourteen ASP preparations from larvae that expressed both Btl:Cherry and Tkv:GFP driven by *btl-Gal4*.

Figure S1

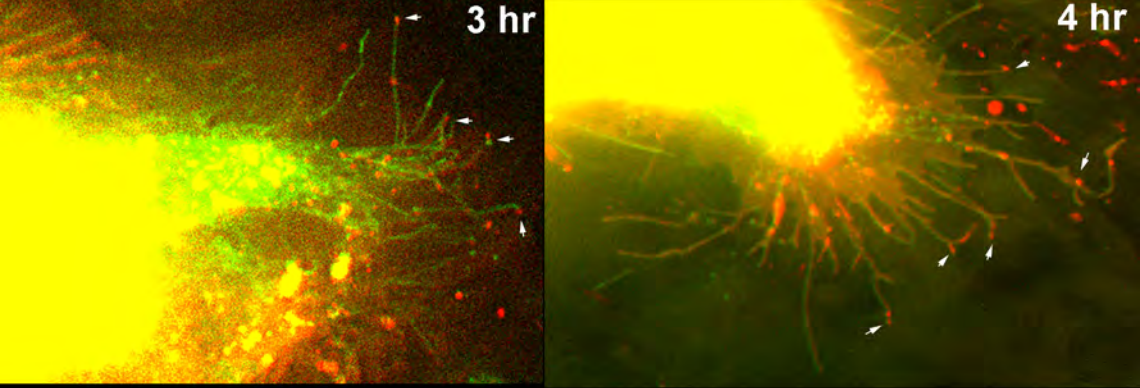


Supplemental Figure 1. Imaginal disc and tracheal cytonemes. (A) Wing disc columnar cell clone extending long apical cytoneme toward (direction indicated by arrow) A/P signaling center. (B) Basal cytonemes from a wing disc columnar cell clone extending with A/P orientation toward the lateral flanks of the disc. (C) Peripodial cell clones in wing. (D) Myoblast clone in wing disc. (E) Peripodial cell clones in eye discs. (F) Eye disc clones marked by CD8:GFP 8 hr after pulse of heat shock that induced cSpi (arrow indicates direction to MF). (G) Eye disc clone co-expressing CD8:GFP and EGFR (arrow indicates direction to MF). (H) Eye disc clones co-expressing CD8:GFP and EGFR (arrow indicates direction to equator). (I) fluorescence and brightfield image of a typical preparation showing wing disc with its associated tracheal branches (expressing CD8:GFP driven by *btl-GAL4*). (J, K) Higher magnification images of boxed regions from (J) showing short cytonemes in TC (J) and cytonemes at tip of ASP (K). Scale bars: 5 μm.

Figure S2

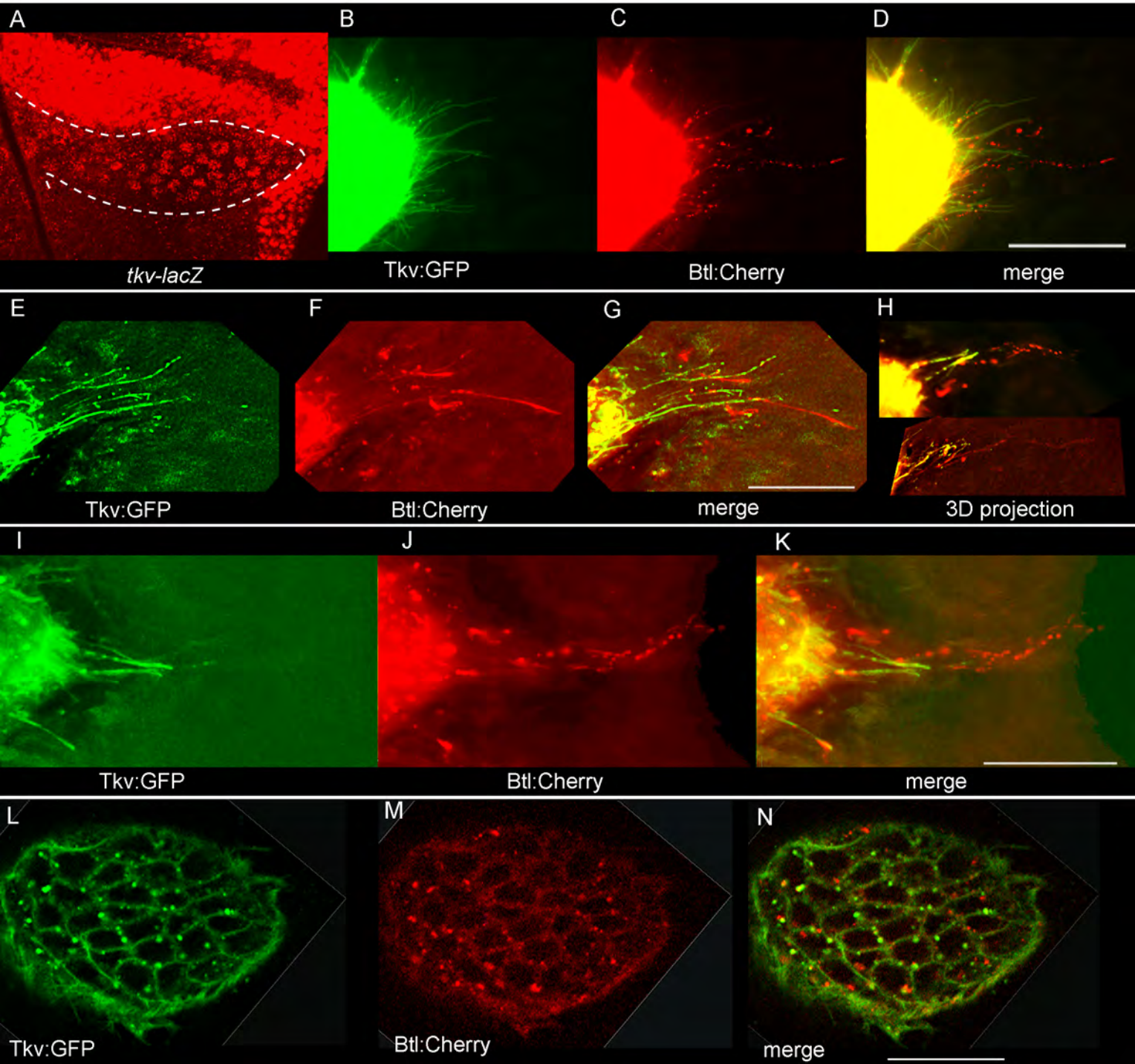


Supplemental Figure 2. Effects of over-expression of signaling proteins on tracheal cytonemes. Representative preparations from larvae expressing CD8:GFP driven by *btl>Gal4* pre- and post- heat shock induction of Hh, Spi, Dpp and Bnl. Scale bars: 30 μm.


3 hr
4 hr

Supplemental Figure 3. Distribution of Btl:Cherry puncta in cytonemes after over-expression of Bnl. Changes to cytonemes at the ASP tip and to ASP tip morphology monitored after heat shock induced expression of Bnl at 3 and 4 hrs post induction. Arrows indicate cytonemes containing Btl:Cherry puncta. Scale bar: 30 μm.

Figure S4


A
B
C
D
tkv-lacZ
Tkv:GFP
Btl:Cherry
merge
E
F
G
H
Tkv:GFP
Btl:Cherry
merge
3D projection
I
J
K
Tkv:GFP
Btl:Cherry
merge
L
M
N
Tkv:GFP
Btl:Cherry
merge

Supplemental Figure 4. Distributions of Tkv:GFP and Btl:Cherry in the ASP. (A) Expression of *tkv-lacZ* in the ASP (outlined by dashed white line) is indicative of Dpp signal transduction. (B-N) Images of an ASP tip from larvae that expressed Tkv:GFP and Btl:Cherry driven by *btl-Gal4*. (B-K) Projection images show that each fluorescent protein illuminates a distinct set of cytonemes. (L-N) Optical section of ASP layer closest to disc epithelium showing Tkv:GFP and Btl:Cherry diffusely distributed in the plasma membrane of ASP cells as well as in discrete puncta. Scale bars: 30 μm.