GPI-anchored FGF directs cytoneme-mediated bidirectional contacts to regulate its tissue-specific dispersion.

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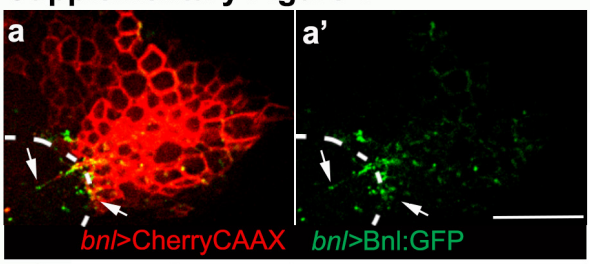
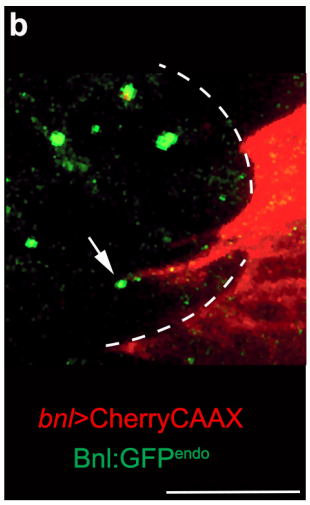
Contents

Supplementary Figure 1





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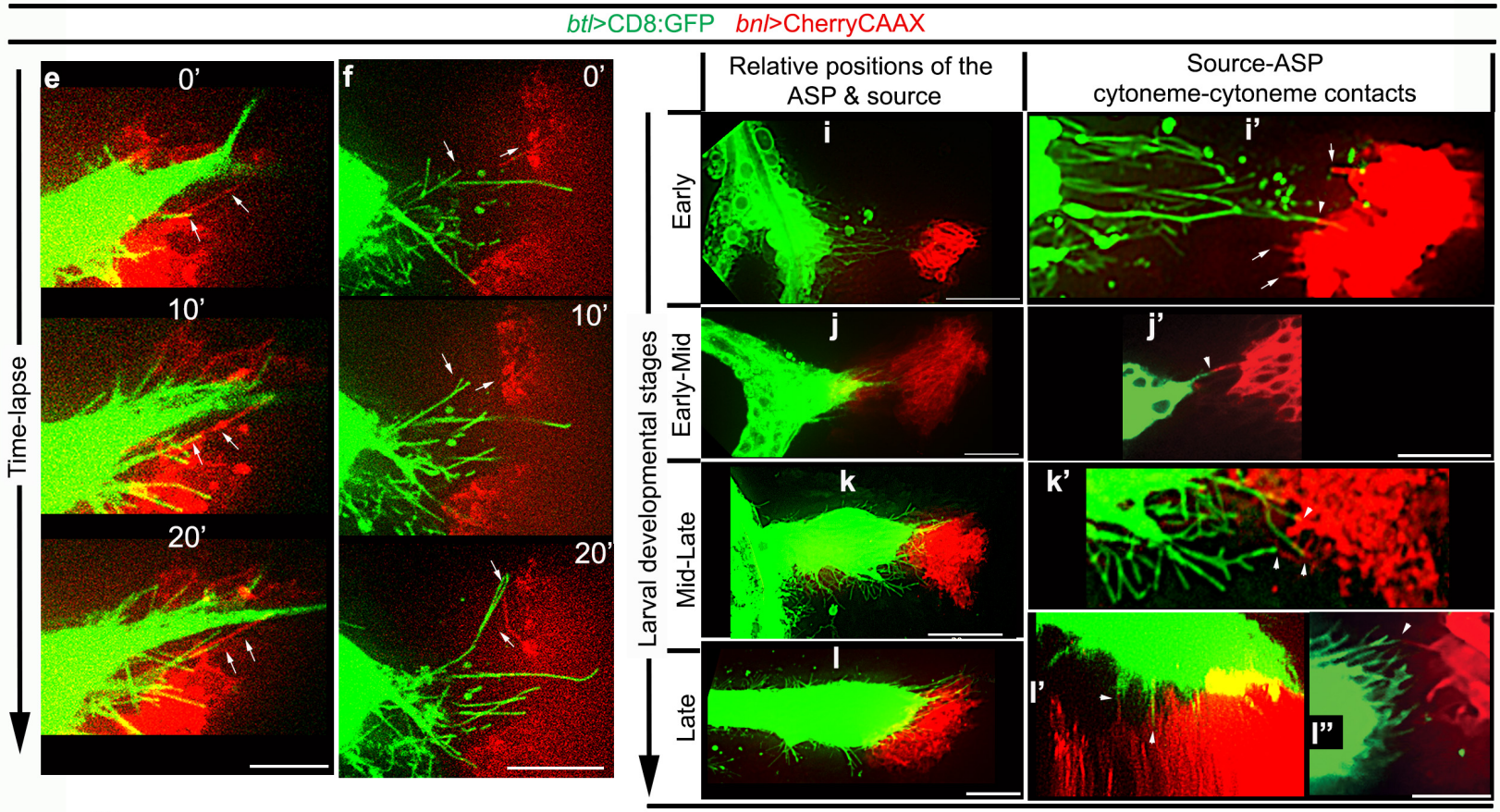
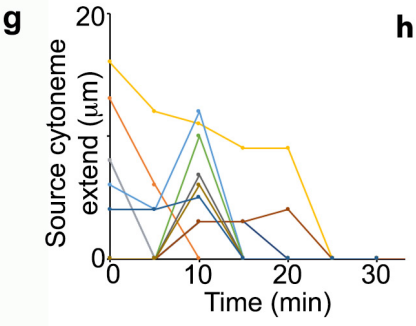
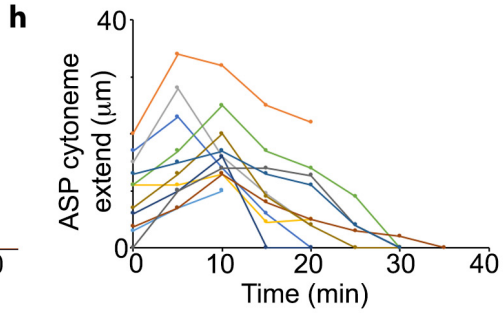
	Sample #	Av. # cytonemes	
		ASP (*btl-Gal4; UAS-CD8:GFP*)	Disc *bnl* source (*bnl-LexA; lexO-CherryCAAX*)
Live tissue	5	31.6±1.7	7.6±2.5
Fixed tissue	5	11.2±2.8	2±1

d

# of clones	# of cells in clones	# of cytonemes	# of cytonemes/cell
1	1	2	2
2	1	2	2
3	5	19	3.8
4	2	7	3.5
5	1	5	5
6	1	5	5
7	2	6	3
8	8	20	2.5
9	2	16	8
10	2	2	1
11	2	7	3.5
12	2	5	2.5
13	1	8	8
Average	2.3	8	3.8

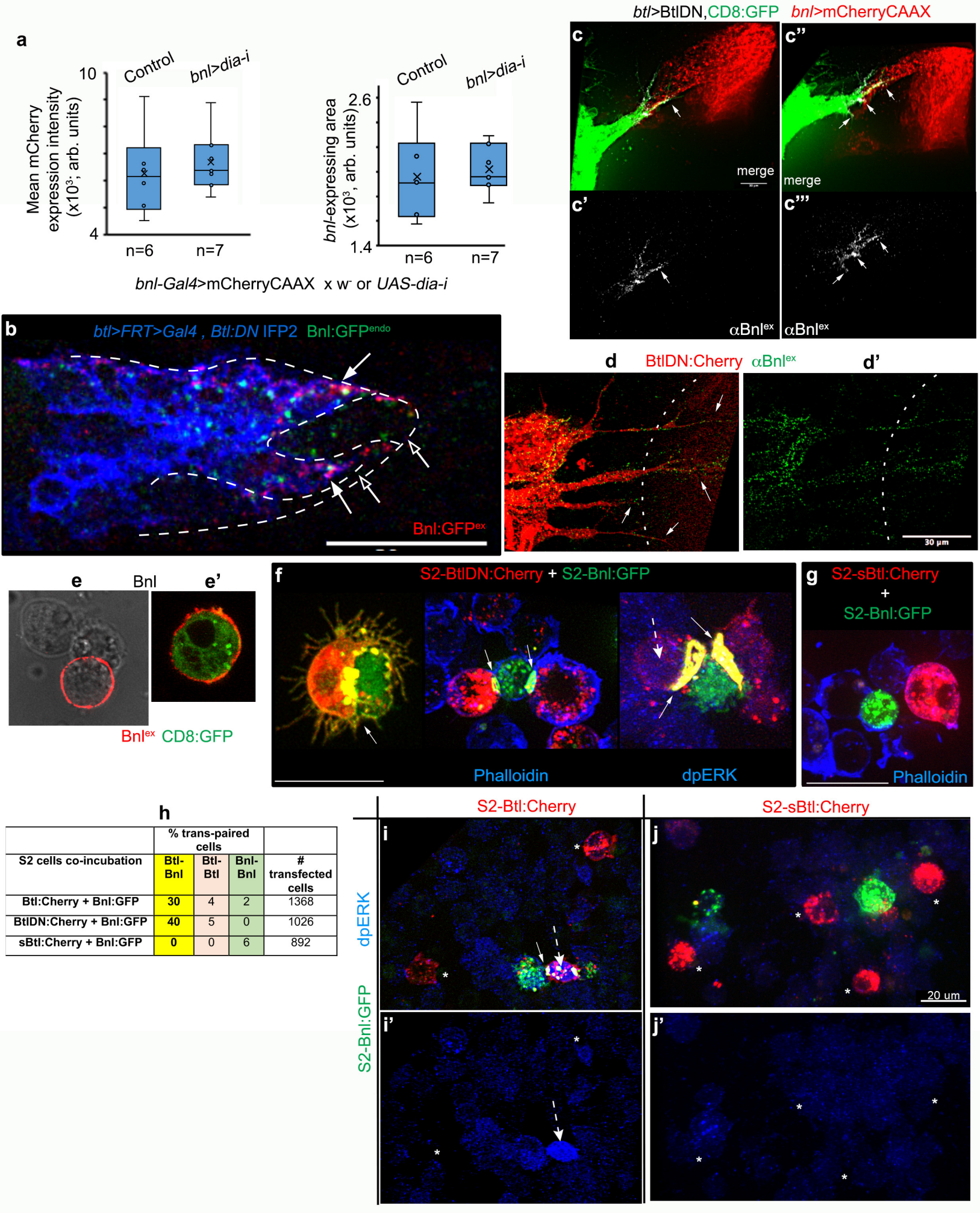






Supplementary Figure 1. Bnl sending and receiving cytonemes reciprocally guide each other. **a-b** Live images of mCherryCAAX-marked wing disc *bnl* source (red) expressing either Bnl:GFP by *bnl-Gal4* (a,a'; *UAS-mCherryCAAX; bnl-Gal4* X *UAS-Bnl:GFP*) or endogenous Bnl:GFP^{endo} (b; *bnl:gfp^{endo}/bnl>mCherryCAAX*; spinning disc confocal), showing ASP (dashed line)-specific polarity of Bnl:GFP presentation through cytonemes (arrows). **c** A comparison of cytoneme numbers (average ± S.D.) from the ASP and wing disc source under live and fixed imaging conditions, showing that the source cytonemes are detected mostly in live imaging. **d** Table showing the number of source cytonemes emanating from CD8:GFP-marked clones within the *bnl*-source (see Methods and Fig. 1h-h''). **e-h** Time-lapse images, showing repeated cycles of extension and retraction of source (red) and recipient (green) cytonemes for reciprocal contacts; g,h, Line plots showing interacting source and recipient cytoneme dynamics (also see Supplementary Table 1); the same color in g and h represents a pair of interacting source and ASP cytonemes. **i-l''** Maintenance of a convergently polarized cytoneme-forming niche at the ASP:source interface throughout the larval development. **e-l''** genotype - *btl-Gal4,UAS-CD8:GFP/+*; *bnl-LexA,lexO-mCherryCAAX/+*. Scale bars, 20 μm. Source data are provided as a Source Data file.

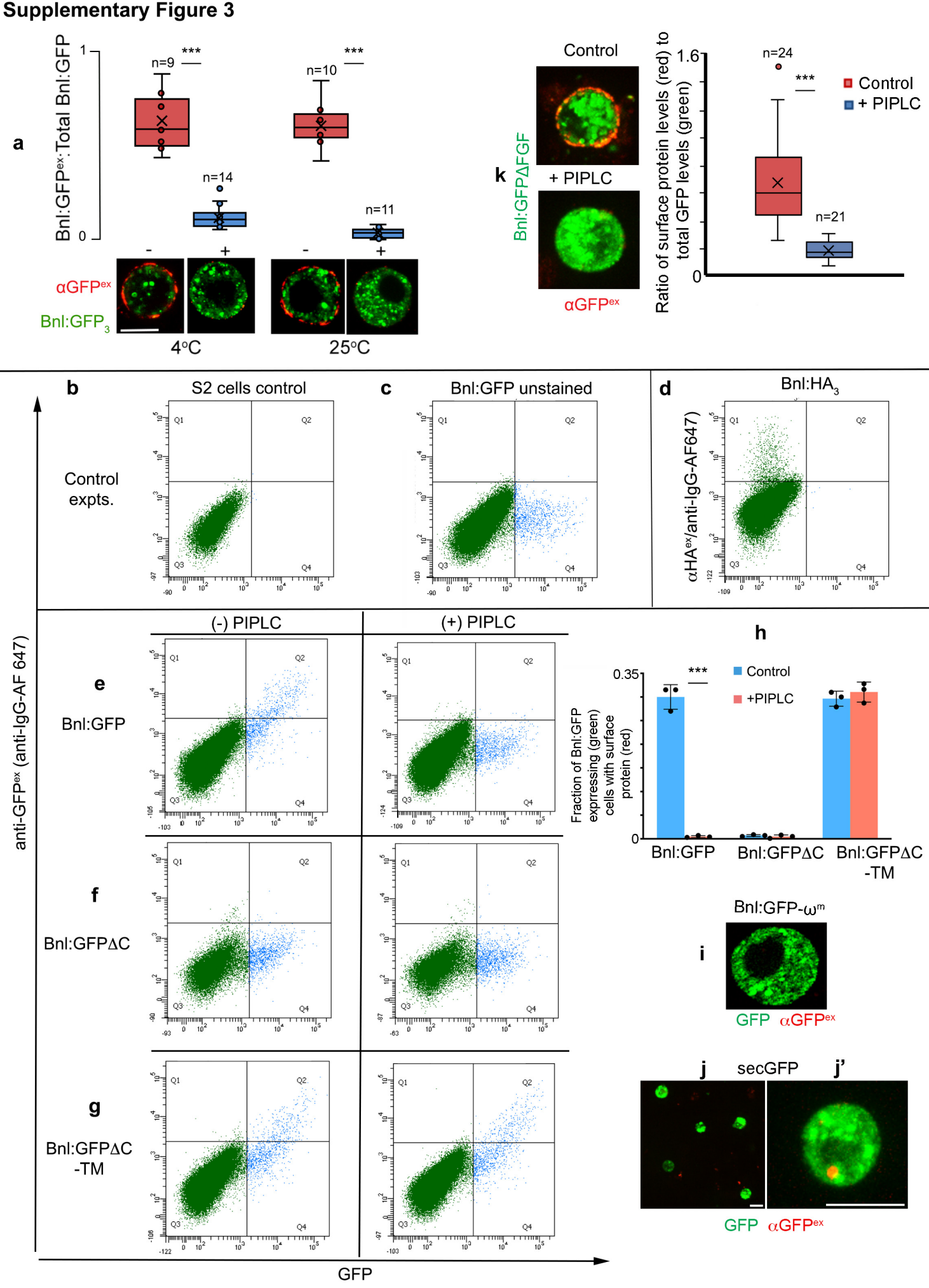
Supplementary Figure 2



S2 cells co-incubation	% trans-paired cells: Btl-Bnl	Btl-Btl	Bnl-Bnl	# transfected cells
Btl:Cherry + Bnl:GFP	30	4	2	1368
BtlDN:Cherry + Bnl:GFP	40	5	0	1026
sBtl:Cherry + Bnl:GFP	0	0	6	892

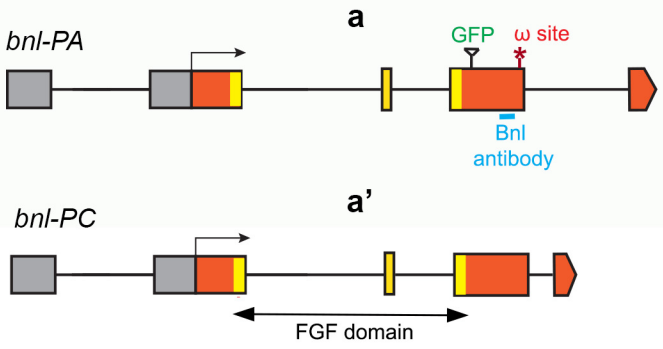
Supplementary Figure 2. CAM-like Btl-Bnl binding mediates reciprocal contact formation.
a *dia-i* expression under the *bnl-Gal4* control did not change *bnl* expression area or levels as detected by the mCherryCAAX expression (*bnl-Ga4, UAS-mCherryCAAX x UAS-dia-i* (for control, *w-* was crossed to *bnl-Ga4, UAS-mCherryCAAX*); in box plots, box shows the median as well as 1st quartile and 3rd quartile, and whiskers are minimum and maximum; n - biologically independent sample number; p values - unpaired two-tailed t-test; p=0.59 for mean mCherry expression intensity and p=0.72 for *bnl*-expressing area. **b** A mosaic ASP with Btl:DN-expressing IFP2-marked clones in the *bnl:GFP^{endo}* knock-in background (see Methods); Btl:DN-expressing surface areas on the ASP showed increased Bnl:GFP^{endo} reception from the wing disc (arrow; probed by αGFP^{ex}) compared to the WT areas (unmarked) of the same ASP tip (open arrow). **c-c'''** Bnl^{ex} (grey, αBnl^{ex}) is asymmetrically enriched (arrow) at the contact sites between source and Btl:DN-expressing ASP projections or cytonemes; c''/c''', 3D projection of c/c'. **d,d'** Btl-DN:Cherry-containing cytonemes (arrow) emanating from a rudimentary ASP localized Bnl^{ex} (green, αBnl^{ex}) puncta on their surfaces; dashed line, source area. **e,e'** αBnl^{ex}-stained S2 cells expressing either Bnl (e) or Bnl and CD8:GFP (e'; *act-Gal4*, *UAS*-Bnl, *UAS*-CD8:GFP), showing surface localized Bnl^{ex}, exclusively on the producing cell. **f** Different trans-paired forms of S2-Bnl:GFP and S2-Btl:DN:Cherry; arrow, trans-synaptic receptor-ligand co-clusters; dashed arrow, absence of nuclear dpERK in trans-paired S2-Btl:DN:Cherry. **g** Absence of trans-paring between S2-sBtl:Cherry and S2-Bnl:GFP. **h** Relatively high frequency of heterotypic Btl-Bnl trans-pairing in comparison to homotypic Btl-Btl or Bnl-Bnl trans-pairing in S2-Btl:Cherry variants/S2-Bnl:GFP co-incubation assays. **i-j'** Representative examples of αdpERK-stained (blue) image frames, comparing trans-pairing experiments between S2-Bnl:GFP/S2-Btl:Cherry (i,i') and S2-Bnl:GFP/S2-sBtl:Cherry (j,j'); arrow, trans-synaptic Btl-Bnl co-cluster; dashed arrow, nucleus in trans-adhered Btl-expressing cells; *, receptor-expressing cells lacking dpERK. Scale bars, 20 μm, 30 μm (b,d,d'). Source data are provided as a Source Data file.

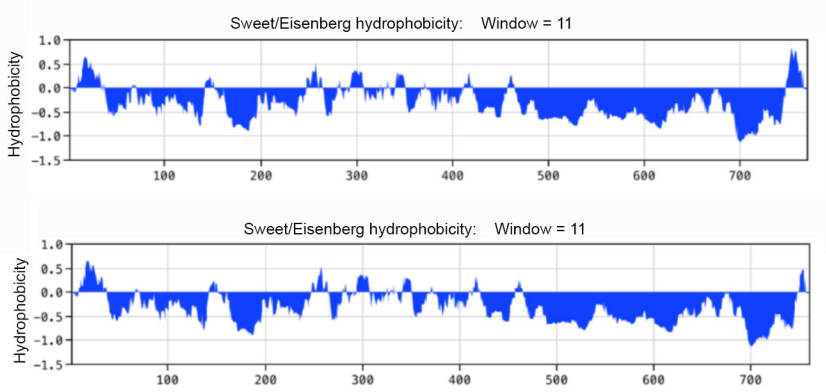
Supplementary Figure 3

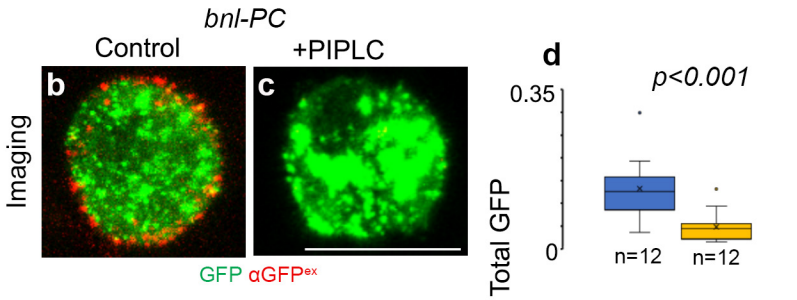


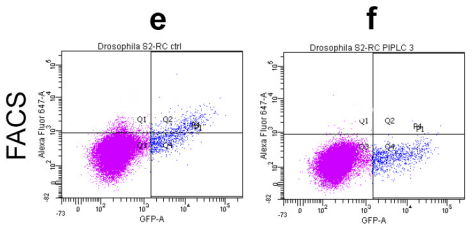
Supplementary Figure 3. A GPI anchor tethers Bnl to the source cell surface.
a-h S2 cells co-transfected with *actin-Gal4* and *UAS*-X; (X = Bnl:HA_3, Bnl:GFP_3, BnlHA_1:GFP_3 (Bnl:GFP), Bnl:HA_1:GFP_3ΔC (Bnl:GFPΔC), or Bnl:HA_1:GFP_3ΔC-TM (Bnl:GFPΔC-TM) as indicated; cells were surface immune-stained either with HA or GFP antibodies as indicated. a, Box plots depicting the ratio of surface localized Bnl:GFP_3 (red, αGFP^{ex} immunostaining) to total Bnl:GFP_3 (green) in S2 cells before (-) after (+) the PIPLC treatment at various temperatures; lower panels, representative images of S2 cells as indicated; box shows the median as well as 1st quartile and 3rd quartile, and whiskers are minimum and maximum; n represents # cells examined; p values were calculated using unpaired two-tailed t-test; ***, p<0.001. **b-g** Representative flow cytometry profiles of S2 cells (b, control) or S2 cells expressing various constructs as indicated; b,c,d, FACS control; d, surface αHA^{ex} staining for Bnl:HA_3 as a control profile for GFP-positive cells. **h** Bar graphs comparing mean values (± SD) obtained by flow cytometry analyses of cells from three independent transfection experiments similar to e, f, g; ***, p<0.001 (unpaired two-tailed t-test); total number of GFP+ cells: 3670 (Bnl:GFP, control), 3095 (Bnl:GFP, +PIPLC), 3240 (Bnl:GFPΔC, control), 3044 (Bnl:GFPΔC, +PIPLC), 3000 (Bnl:GFPΔC-TM, control), 3000 (Bnl:GFPΔC-TM, +PIPLC). **i** Lack of surface-localized protein (red, probed with αGFP^{ex}) of Bnl:GFP-ω^m expressed in S2 cells. **j,j'** αGFP^{ex} immunostained S2 cells expressing secGFP construct showing the lack of surface distribution of the proteins due to its immediate secretion [1]. This is a control for bGFP-GPI, which is the same secGFP with Bnl's C-terminal signal sequence, leading to its GPI-anchoring to the producing cell surface (see Figure 4d'-f). **k** αGFP^{ex} immunostained S2 cells (left panels) expressing Bnl:GFPΔFGF construct showing PIPLC-sensitive surface distribution of the protein; right panel, box plots comparing the fraction of expressed protein on cell surface with and without PIPLC treatment; box shows the median as well as 1st quartile and 3rd quartile, and whiskers are minimum and maximum; n = number of cells as indicated; ***, p<0.001; p values were calculated using unpaired two-tailed t-test. Scale bars, 10 μm. Source data are provided as a Source Data file.

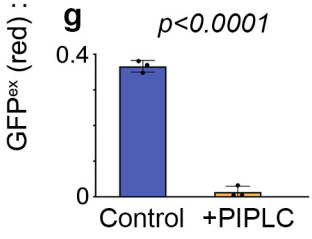
Supplementary Figure 4





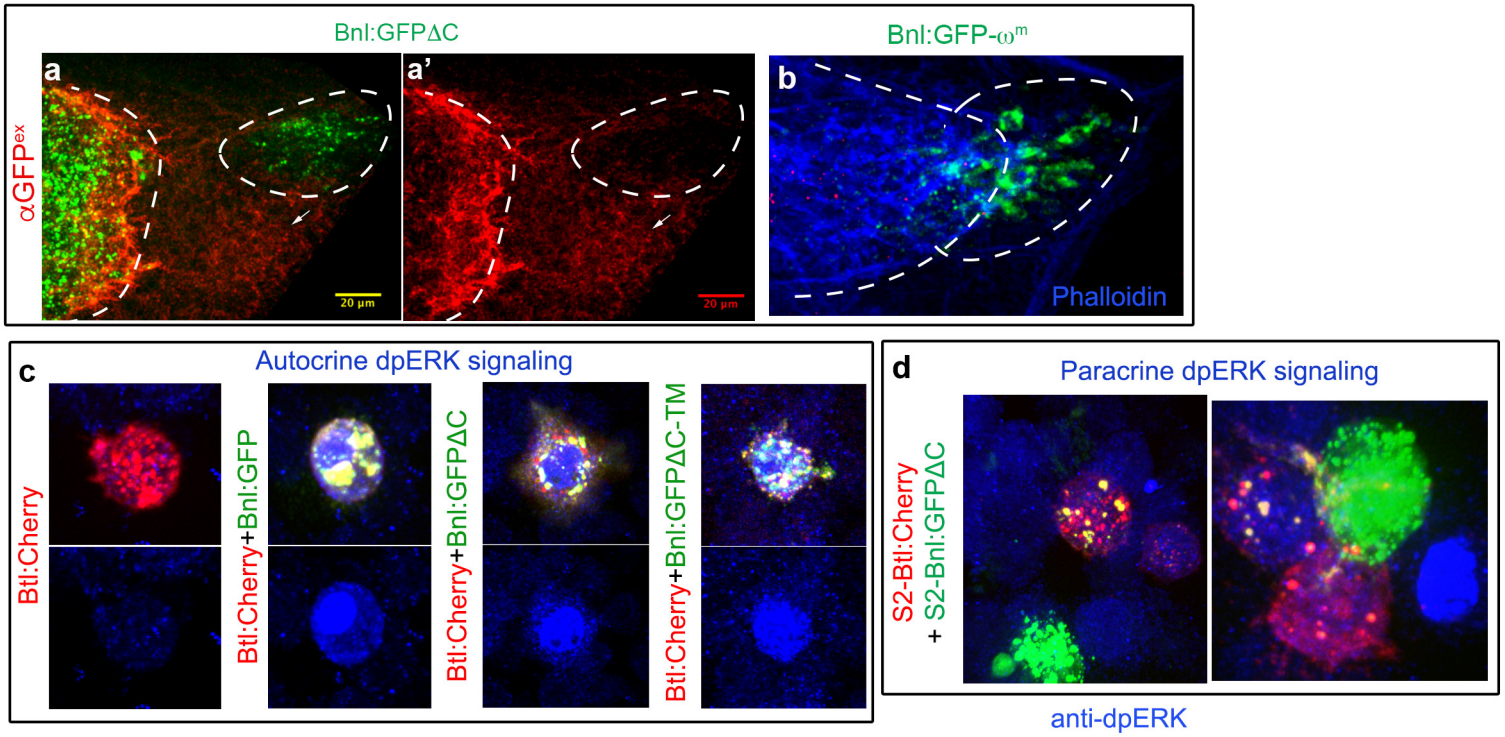






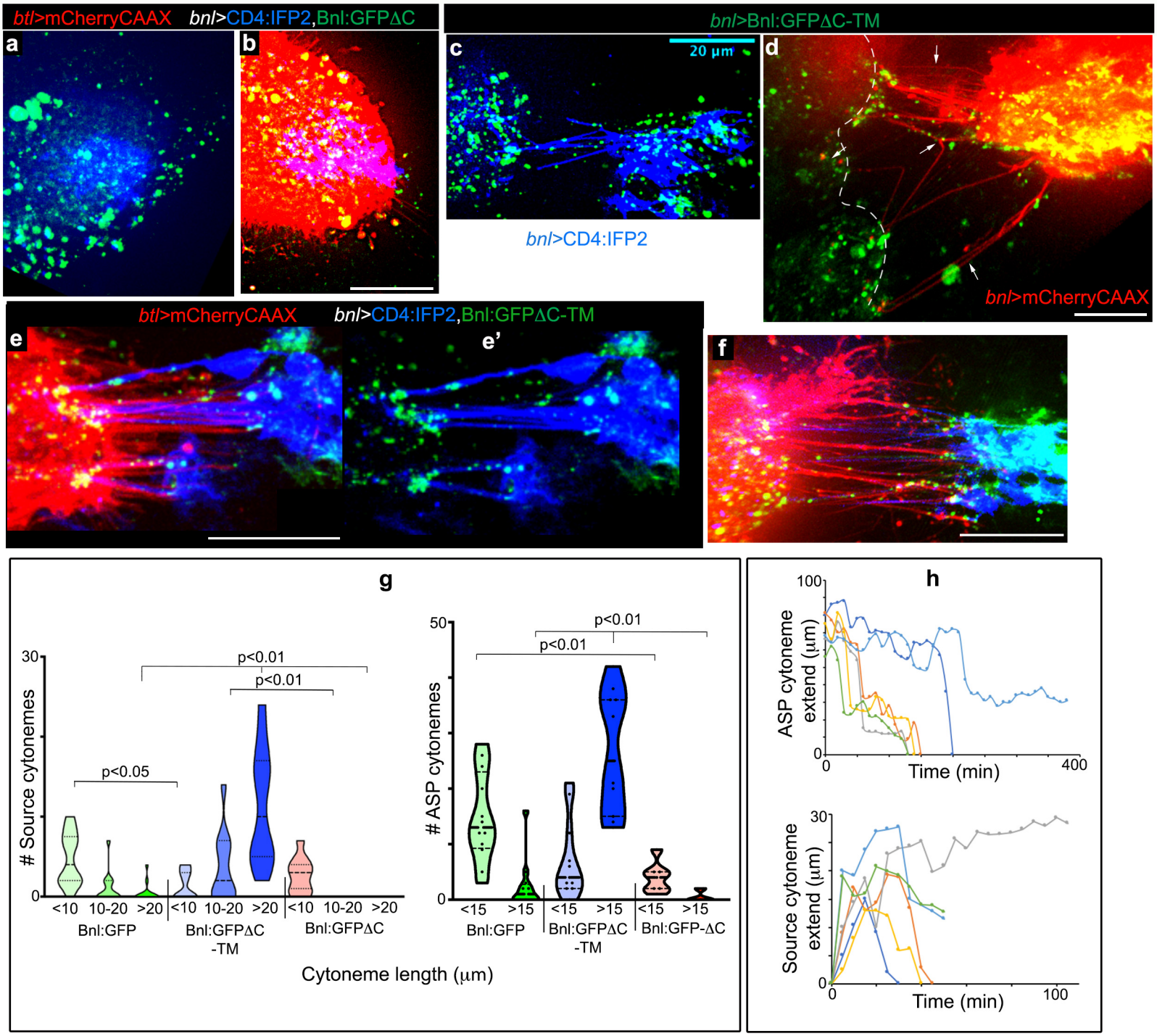
Supplementary Figure 4. Characterization of GPI-anchored Bnl-PC isoform.
a-a''' Comparison of PA and PC splice variants of *bnl*: **a,a'** Schematic maps of PA and PC loci highlighting identical FGF signaling domain, Bnl antibody binding site used for probing Bnl^{ex}, GFP tag (for probing $Bnl:GFP^{endo}$), putative ω-site; Grey box, non-coding exons, colored box, coding exons. However, due to the alternative splicing of the last coding exons, *bnl-PC* isoform is 11 amino acid shorter at its C-terminal end than the *bnl-PA.* Subsequent to the common sequence between PA and PC, PC isoform has only 7 amino acids at its C-terminus, which is replaced by 18 amino acids sequences in PA. **a'',a'''** Comparative hydrophobicity plots of PA and PC variants showing a reduced hydrophobic stretch of C-terminal region of PC. **b-g** Extracellular αGFP^{ex}-immunostaining of S2 cells expressing $Bnl:GFP_3$-PC showed surface-localized $Bnl:GFP_3\text{-}PC^{ex}$ (red), which is removed by PIPLC assay; **d** box plots showing surface localized fractions (red, probed with αGFP^{ex}) of total $Bnl:GFP_3$-PC expressed in S2 cells before and after PIPLC treatment; box shows the median as well as 1st quartile and 3rd quartile, and whiskers are minimum and maximum; n represents # cells examined by imaging as shown in b,c; p value was calculated using unpaired two-tailed t-test; p=0.000649. **e,f** flow cytometric analyses of the same; **g** bar graphs showing quantitative values obtained from flow cytometry experiments depicting the average fraction of $Bnl:GFP_3$-PC-expressing cells (GFP positive) containing surface localized $Bnl:GFP_3\text{-}PC^{ex}$ (αGFP^{ex} immunostained (red)) before and after the PIPLC treatment; values represent the mean $\pm$ SD from 3 independent experiments; p value was calculated using unpaired two-tailed t-test; total GFP+ events examined over 3 repeats: 3134 (control) and 2784 (+PIPLC);. Scale bars, 10 μm. Source data are provided as a Source Data file.

Supplementary Figure 5



Supplementary Figure 5. Autocrine and paracrine activity of Bnl variants.
a-b Extracellular distribution of Bnl:GFPΔC (a,a') and Bnl:GFP-ω^m (b), when expressed under *bnl-Gal4* in the wing disc source; αGFP^{ex} immunostaining (red) showing that Bnl:GFPΔC is poorly retained on the source cell surface area (green punctate demarcated by dashed line), but are spread on the extracellular plane of the non-expressing disc cells (only red). ASP had both Bnl:GFPΔC^{ex} and internalized Bnl:GFPΔC (probed only by GFP), showing non-autonomous signal dispersal. In contrast, Bnl:GFP-ω^m (b) is poorly externalized from the source and not received by the ASP (Phalloidin-stained). **c** Efficient autonomous MAPK signaling (nuclear dpERK, blue) of different Bnl:GFP variants when co-expressed with Btl:Cherry in S2 cells. **d** Inefficient non-autonomous MAPK signaling of Bnl:GFPΔC when S2-Bnl:GFPΔC cells were co-incubated with S2-Btl:Cherry cells.

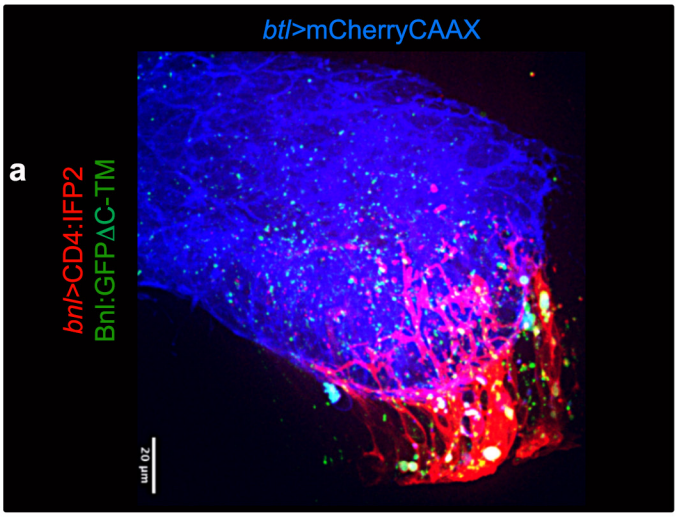
Supplementary Figure 6

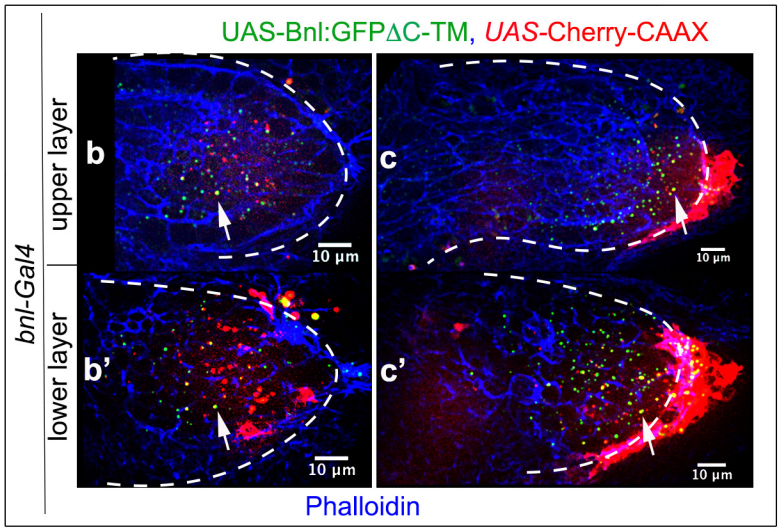


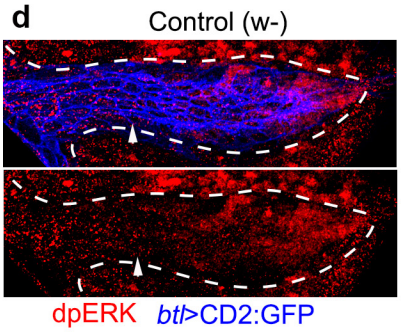
Supplementary Figure 6. GPI-anchored Bnl induces cytoneme-mediated bidirectional matchmaking for contacts.

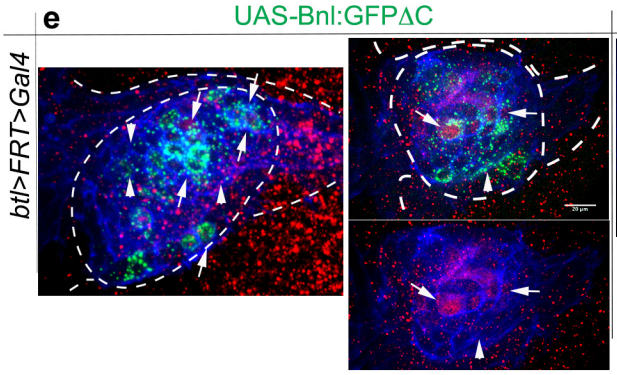
a Split channels of Figure 6g, showing the random spread of Bnl:GFP∆C from its source (blue, *bnl>CD4:IFP2*). **b** An example showing the loss of ASP cytonemes (red), when Bnl:GFP∆C was overexpressed from CD4:IFP2-marked source cells (blue). **c** Split green and blue channels of Figure 6j showing Bnl:GFP∆C-TM-containing source cytonemes contacting the ASP. **d-f** Examples showing CAM-like activity of Bnl:GFP∆C-TM when expressed from the source: (d) Long polarized Bnl:GFP∆C-TM-expressing source cytonemes (arrows) (red, *bnl>mCherryCAAX*) connected to the ASP and disc-associated transverse connective (dashed outline). (e-f) Bundles of ASP and source cytonemes interacting through Bnl:GFP∆C-TM-enriched lateral contact sites; e', split blue and green channels of (e). **g** Violin plots showing a comparison of the number and length distribution of ASP and source cytonemes (from Fig. 6f-l) induced by Bnl:GFP, Bnl:GFP∆C, or Bnl:GFP∆C-TM, when overexpressed from the disc source; in violin plots, black dotted lines show the median as well as 25^{th} and 75^{th} percentiles; n=13 (Bnl:GFP, source), 11 (TM, source), 11 (∆C, source), 12 (Bnl:GFP, ASP), 11 (TM, ASP), 7 (∆C, ASP) biologically independent samples; p values were calculated using one way-ANOVA followed by Tukey's honestly significant different test. **h** Line plots showing dynamics of the source and recipient cytonemes as indicated when Bnl:GFP∆C-TM was expressed in *bnl* source using *bnl-Gal4* (see Supplementary Table 1); the same color represents the interacting Bnl-receiving and -sending cytonemes from the same sample. All panels, live imaging. Scale bars, 20 μm. Source data are provided as a Source Data file.

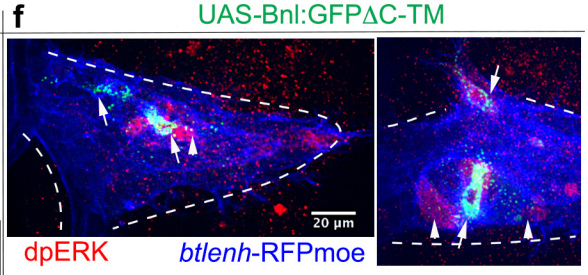
Supplementary Figure 7











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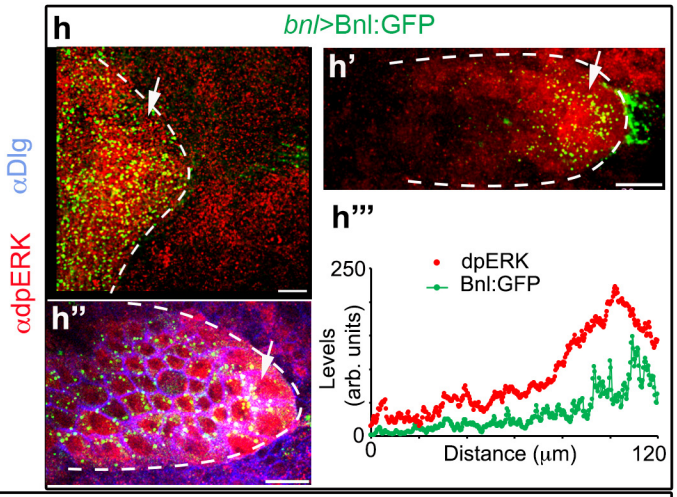
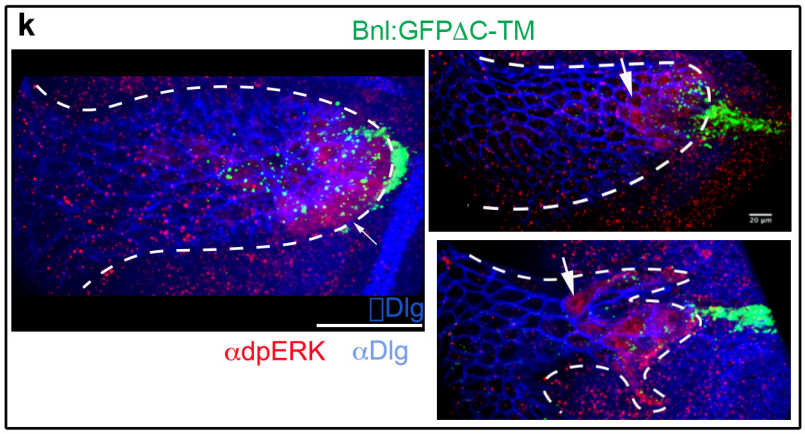
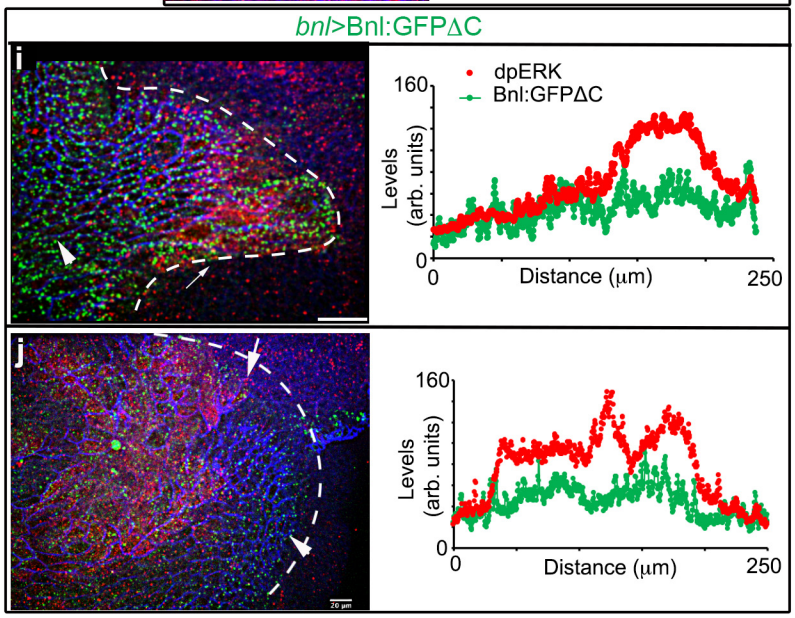
Genotype *hsflp/+;* *btlenh>FRT>Gal4,* *btlenh-RFPmoe/UAS-"X"*	Sample #	Dispersal range (# cells)	% signal receiving ASP cells with nuclear dpERK	% clones induced growth	% clones induced branching
Bnl:GFP	24	2.8±0.7	99.2±2.3	83	25
Bnl:GFP-TM	33	2.3±0.8	82.9±19.9	76	36
Bnl:GFP-ΔGPI	21	4.2±0.9	36.9±27.4	81	0







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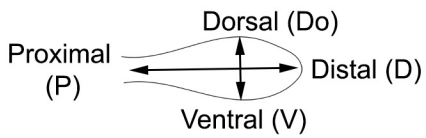


Genotype	Sample #	Av. longest Do-V axis length	Av. longest D-P axis length	Ratio Do-V/D-P
bnl:GFP^{endo} *	9	33±4	119±10	0.28±0.04
bnlGal4>Bnl:GFP	7	95±24	120±14	0.82±0.3
bnlGal4>Bnl:GFP-TM	15	56±11	137±10	0.41±0.07
bnlGal4>Bnl:GFPΔGPI	8	146±11	104±24	1.50±0.47

Supplementary Figure 7. GPI anchoring is required for Bnl release and morphogen-like signaling. **a** Strong affinity and adhesion of Bnl:GFPΔC-TM-expressing source cytonemes (red, *bnl>CD4:IFP2*) with the ASP surface (blue, *btl>mCherryCAAX*). **b-c'** Images of two wing discs expressing Bnl:GFPΔC-TM and mCherryCAAX from the *bnl* source (red), showing endocytosed Bnl:GFPΔC-TM puncta colocalized with the source membrane in upper and lower layer cells of the tubular ASP epithelium; Phalloidin-Alexa Fluor 647 (blue) marked cell outlines. **d** Control (*w-*) ASP showing the lack of nuclear dpERK in the ASP stalk and TC region, where the GOF clones were scored (see Fig. 9a-d). **e-g** Examples of ASPs with ΔC or TM GOF clones (1-2 cell size) (arrows) and their non-autonomous signaling (arrowhead; red, dpERK). **g** Table showing non-autonomous effects of Bnl:GFP, TM, and ΔC GOF clones in the ASP stalk; sample # (n) represents the number of clones examined over >10 biologically independent samples; values represent the mean ± SD; p values for % signal receiving ASP cells with nuclear dpERK: p <0.01, for Bnl:GFP-ΔC vs. Bnl:GFP or Bnl:GFPΔC-TM; p <0.05, for Bnl:GFP vs. Bnl:GFPΔC-TM; p values were calculated using one way-ANOVA followed by Tukey's honestly significant different test. **h-l** Comparative analyses of the activity of Bnl:GFP, Bnl:GFPΔC-TM (TM), and Bnl:GFPΔC (ΔC), expressed from wing disc source under *bnl-Gal4* (*bnl-Gal4 X UAS-X*)**:** (**h-h'''**) Wing discs expressing Bnl:GFP, showing a spatial coordination of signal distribution (green puncta), signaling patterns (dpERK, red), and ASP growth. (**i-k**) The coordination between signal distribution, signaling, and growth was uncoupled by ΔC expression and was regained with TM. However, TM distribution was restricted in range in comparison to Bnl:GFP. (**l**) Comparison of ASP shapes in conditions as indicated; *, homozygous *bnl:gfp*^{endo} larvae used as the control for overexpressed Bnl:GFP variants; top panel, illustration showing the measurement of the longest Do-V and D-P axes (μm) from extended Z-projected ASP images; sample # (n) represents the number of biologically independent samples; values represent the mean ± SD; p values: Do-V/D-P axes: p <0.01, *bnl:gfp*^{endo} vs. Bnl:GFP or ΔC and Bnl:GFP vs. TM or ΔC; p values were calculated using one way-ANOVA followed by Tukey's honestly significant different test. h-k, arrows, recipient cells with signaling; arrowhead, recipient cells without signaling; αDlg (blue), cell outlines. All panels, dashed line shows ASP outline or ectopic tracheal outgrowth. Scale bars, 20 μm; 10 μm (b-c'). Source data are provided as a Source Data file.

Supplementary Tables

Supplementary Table 1. Quantification of the dynamics of the interacting cytonemes from Bnl-receiving and -sending cells.

	FGF-receiving cells			FGF-sending cells		
	WT	*bnl>Bnl:GFP*	*bnl>Bnl:GFP ΔC-TM*	WT	*bnl>Bnl:GFP*	*bnl>Bnl:GFP ΔC-TM*
Lifetime (min)	19.55 ±6.1	17.5±5*	180±101.39	7.73± 4.67	ND	46.67±30.11
# Fluctuating peaks/lifetime	1±0	1±0	4.33±2.66	1±0	ND	2.33±1.37
Maximum extension (μm)	19.36 ±7.4	15.8±3.8	76.67±8.98	8.35±4.04	ND	26.52±7.3
Average extension rate (μm/min)	1.28 ±0.79	0.85±0.32	0.47±0.15	1.13±0.57	ND	0.70±0.23
Average retraction rate (μm/min)	1.37 ±0.75	0.93±0.19	0.88±0.22	1.32±0.56	ND	0.79±0.26

Note: Maximum extension - the maximum length of a cytoneme during its lifetime. Peak - Each extension and retraction cycle within the lifetime of a cytoneme. Number of fluctuating peaks/lifetime - the number of extension and retraction cycles of a cytoneme. Average extension and retraction rates - measured by the net cytoneme length change/time during its extension or retraction.

*, For this condition, 4 long cytonemes were used. Most cytonemes (N=25) were short in length and had lifetime <10 min and was not counted in 10 min interval time-lapse movies.

Values represent mean ± SD. N=11 cytonemes for WT; 6 cytonemes for *bnl>Bnl:GFPΔC-TM* receiving and sending cytonemes; 4 cytonemes for *bnl>Bnl:GFP* receiving cytonemes. p values (WT vs *bnl>Bnl:GFPΔC-TM*) for receiving cytoneme dynamics: lifetime, p <0.0001; # fluctuating peaks/lifetime, p <0.001; maximum extension, p <0.0001; average extension rate, p =0.025; average retraction rate, no significant difference. p values for sending cytoneme dynamics: lifetime, p <0.001; # fluctuating peaks/lifetime, p =0.0046; maximum extension, p <0.001; average extension rate, no significant difference; average retraction rate, p =0.047. p values were calculated using unpaired two-tailed t test. p < 0.05 is considered significant. Source data are provided as a Source Data file.

Genotypes: WT: *btlGal4,UAS-CD8:GFP/+;bnlLexA,LexO-mCherryCAAX/+*.
bnl>Bnl:GFP: *btlLexA,LexO-mCherryCAAX/UAS-CD4:mIFP; bnlGal4/UAS-Bnl:GFP.*
bnl>Bnl:GFPΔC-TM: *UAS-Bnl:GFPΔC-TM/UAS-CD4:mIFP; bnlGal4/btlLexA,LexO-mCherryCAAX* for FGF-receiving cytonemes, and *UAS-mCherryCAAX/UAS-Bnl:GFPΔC-TM; bnlGal4/+* for FGF-sending cytonemes.

Supplementary Table 2. Comparison of the ASP and source cytoneme numbers when *diaRNAi* was expressed in the ASP.

		# ASP cytonemes < 15 μm	# ASP cytonemes > 15 μm	# FGF source cytonemes
Control (N=11)	Average	11.55	17	9.45
	SD	±4.57	±4.96	±3.30
btlGal4>diaRNAi (N=8)	Average	10	1	0
	SD	±7.66	±1.06	±0.35
p (unpaired t-test)		0.559367458	5.87525E-08	4.27454E-07

Note: Values represent mean ± SD. N represents the number of biologically independent samples. p values were calculated using unpaired two-tailed t test. Source data are provided as a Source Data file.
Genotypes: Control: *btlGal4,UAS-CD8GFP/+;bnlLexA,LexO-mCherryCAAX/+.*
btl-Gal4>diaRNAi: *btlGal4,UAS-CD8GFP/tub-Gal80^{ts};bnl-LexA,LexO-mCherryCAAX/ UAS-diaRNAi.*

Supplementary Table 3: Autocrine and paracrine MAPK signaling activity of Bnl:GFP variants in S2 cells

a. Autocrine activity: S2-Btl:Cherry co-expressed with Bnl:GFP variants

S2 cell co-expression	# total Btl cells	# Btl cells with nuclear dpERK	% Btl cells with nuclear dpERK
Bnl:GFP+Btl:Cherry	15	14	93
Bnl:GFPΔGPI+Btl:Cherry	22	21	95
Bnl:GFPΔGPI-TM +Btl:Cherry	16	15	94

b. Paracrine activity: S2-Bnl:GFP variants co-incubated with S2-Btl:Cherry

S2-Btl:Cherry cells (# or %)	Bnl:GFP +Btl:Cherry	Bnl:GFPΔGPI +Btl:Cherry	Bnl:GFPΔGPI-TM +Btl:Cherry
# of total cells (A)	169	188	123
# of (A) with dpERK	76	65	60
% of (A) with dpERK	44.9	34.6*	48.8
# of trans-paired cells with S2-Bnl:GFP/TM or adjacent to S2-ΔC (B)	78	33	87
# of (B) with dpERK	71	5	58
% of (B) with dpERK	91	15**	67
# of uncoupled Btl +ve cells (C)	91	188	36
# of (C) with dpERK	5	60	2
% of (C) with dpERK	5	32 **	6

Note: data represents results from three independent transfection repeats.

Supplementary Table 4: Resources and reagents used in this study

REAGENT or RESOURCE	DESCRIPTION	SOURCE
Antibodies		
Mouse monoclonal anti-Discs large (Dlg)	1:100	DSHB, Cat# 4F3 anti-discs large; RRID: AB_528203
Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) rabbit monoclonal antibody (dpERK)	1:250 in tissue and 1:1000 in S2 cells	Cell signaling Technology; Cat# 4370; RRID: AB_2315112
Rat monoclonal anti-HA (3F10)	1:1000 for standard and 1:500 for EIF	Roche; Cat#11867423001; RRID: AB_390918
Rabbit polyclonal anti-Bnl	1:500 for EIF [1]	N/A
Rabbit anti-GFP antibody	1:3000 for EIF	Abcam; Cat# ab6556; RRID: AB_305564
Goat anti-Mouse IgG (H+L), Alexa Fluor 555	1:1000	Thermo Fisher Scientific; A21434
Goat anti-Mouse IgG (H+L), Alexa Fluor 647	1:1000	Thermo Fisher Scientific; A28181
Goat anti-Rat IgG (H+L), Alexa Fluor 647	1:1000	Thermo Fisher Scientific; A21247
Goat anti-Rabbit IgG (H+L), Alexa Fluor 555	1:1000	Thermo Fisher Scientific; A21428
Goat anti-Rabbit IgG (H+L), Alexa Fluor 647	1:1000	Thermo Fisher Scientific; A21244
Bacterial and Virus Strains		
DH5 Alpha		
Chemicals, Peptides, and Recombinant Proteins		
Alexa Fluor 647 Phalloidin	Thermo Fisher Scientific	Cat# A22287, RRID: AB_2620155
Furin Inhibitor I - Calbiochem	Sigma-Aldrich	Cat# 344930
Furin Inhibitor II - Calbiochem	Sigma-Aldrich	Cat# 344931
Phospholipase C, Phosphatidylinositol-specific from *Bacillus cereus*	Invitrogen	Cat# P6466
Critical Commercial Assays		
Lipofectamine 3000 Transfection Reagent	Thermo Fisher Scientific	Cat# L3000008
Mirus TransIT®-Insect Transfection Reagent	Mirus Bio	
TRI Reagent	Sigma-Aldrich	Cat# T9424
One*Taq*® One-Step RT-PCR Kit	NEB	Cat# E5315S
Deposited Data		
Raw data from all the figures	This paper	
Experimental Models: Cell Lines		
D. melanogaster: Cell line S2: S2-DRSC	Laboratory of Thomas B. Kornberg	FlyBase: FBtc0000181
Experimental Models: Organisms/Strains		
D. melanogaster: *UAS-Bnl:GFPΔC*	This paper	N/A
D. melanogaster: *UAS-Bnl:GFPΔC-TM*	This paper	N/A

D. melanogaster: *UAS-Bnl:GFPΔC_{168}-TM*	This paper	N/A
D. melanogaster: *UAS-Bnl:GFPΔC_{168}*	This paper	N/A
D. melanogaster: *LexO-BtlDN:Cherry*	This paper	N/A
D. melanogaster: *UAS-Bnl:GFP$-\omega^{m}$*	This paper	N/A
D. melanogaster: *bnl:gfp^{endo}*	[1]	N/A
D. melanogaster: *btl:cherry^{endo}*	[1]	N/A
D. melanogaster: *UAS-Bnl:GFP*	[2]	N/A
D. melanogaster: *UAS-CD8:GFP*	BDSC	5137
D. melanogaster: *UAS-nlsGFP*	BDSC	4776
D. melanogaster: *UAS-mCherryCAAX*	BDSC	59021
D. melanogaster: *UAS-CD4:mIFP*	BDSC	64182
D. melanogaster: *lexO-mCherryCAAX*	[3]	N/A
D. melanogaster: *UAS-Btl^{DN}*	[4]	N/A
D. melanogaster: *UAS-Bnl*	BDSC	64232
D. melanogaster: *UAS-diaRNAi*	BDSC	33424
D. melanogaster: *UAS-Dia-GFP*	[3]	N/A
D. melanogaster: *UAS-ΔDAD-Dia-GFP*	[3]	N/A
D. melanogaster: *bnl-LexA*	[1]	N/A
D. melanogaster: *bnl-Gal4*	BDSC	112825
D. melanogaster: *btl-Gal4*	[5]	N/A
D. melanogaster: *btl-LHG*	[3]	N/A
D. melanogaster: *hs-FLP; btl>y+>Gal4, btl-mRFP1moe*	[6]	N/A
D. melanogaster: *hs-mFlp*	BDSC	N/A
D. melanogaster: *FlyBow FB2.0*	BDSC	N/A
D. melanogaster: *btl:GFP* fTRG	VDRC	318302
D. melanogaster: *hs-Flp*	BDSC	6
D. melanogaster: *tub-Gal80^{ts}*	BDSC	7108
D. melanogaster: *act>CD2>Gal4*	BDSC	4780
D. melanogaster: *w^{1118}*	BDSC	3605
Oligonucleotides		
Primer for cloning *UAS-Bnl:GFPΔC*: GCCAAGCTTGCATGCCGGTACCTTAGTAGCTCGCATCTTCTAGGGATCC	This paper	N/A
Primer for cloning *UAS-Bnl:GFPΔC-TM:* CCCTAGAAGATGCGAGCTACGACTTCGCCTGTGATATTTACATCTGG	This paper	N/A
Primer for cloning *UAS-Bnl:GFPΔC-TM:* GATGTAAATATCACAGGCGAAGTCGTAGCTCGCATCTTCTAGGGATCC	This paper	N/A
Primer for cloning *UAS-Bnl:GFPΔC-TM:* GCCAAGCTTGCATGCCGGTACCTTAGTGGTAGCAGATGAGAGTGATGATC	This paper	N/A
Primer for cloning *UAS-Bnl:GFPΔC_{168}*: GCCAAGCTTGCATGCCATATATTCTAGATTACTTCTTCTTGCCTCCGTGCTG	This paper	N/A
Primer for cloning *UAS-Bnl:GFPΔC_{168}-TM*: CAGCACGGAGGCAAGAAGAAGgacttcgcctgtgatatttacatctgg	This paper	N/A
Primer for cloning *UAS-Bnl:GFPΔC_{168}-TM*: ccagatgtaaatatcacaggcgaagtcCTTCTTCTTGCCTCCGTGCTG	This paper	N/A
Primer for cloning *UAS-Bnl:HA$_1$GFP$_3$Cherry$_c$*: GTTTTGCTCCGAAAAAGAGCCATCCTGATGGTGAGCAAGGGCGAGGAG	This paper	N/A
Primer for cloning *UAS-Bnl:HA$_1$GFP$_3$Cherry$_c$*: GCTGCTGGTACCTTACTTGTACAGCTCGTCCATGCCG	This paper	N/A

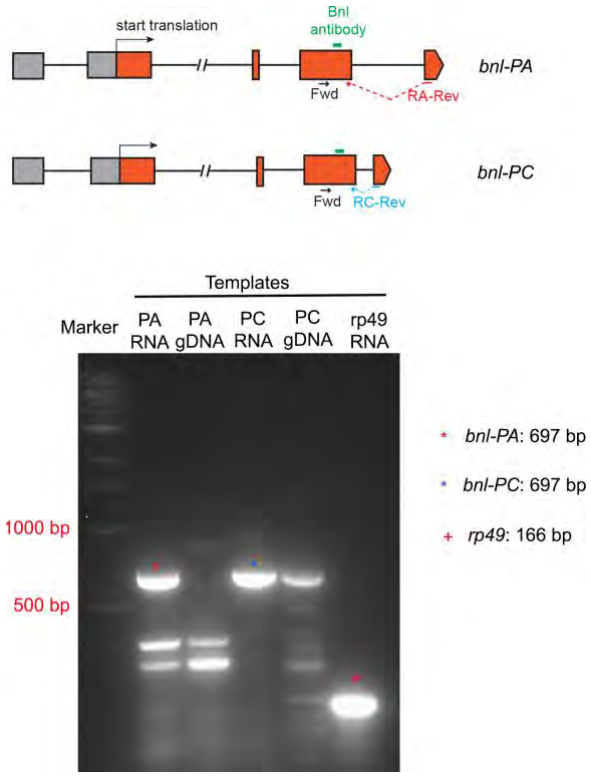
Primer for cloning *UAS-Bnl:GFP-ω^n:* CGAGGCCCAAGGACGCCCCCCACCAGGCGGCGACGATTCG	This paper	N/A
Primer for cloning *UAS-Bnl:GFP-ω^n:* CGAATCGTCGCCGCCTGGTGGGGGGCGTCCTTGGGCCTCG	This paper	N/A
Primer for cloning *UAS-bGFP-GPI:* CAACAACTTGACAATGTCCAAGGGCGAGGAG	This paper	N/A
Primer for cloning *UAS-bGFP-GPI:* GCCCTTGGACATTGTCAAGTTGTTGTCCATGGCC	This paper	N/A
Primer for cloning *UAS-bGFP-GPI:* TGGATGAGCTGTACAAGACCGAGGGCGACGGTG	This paper	N/A
Primer for cloning *UAS-bGFP-GPI:* TCGGTCTTGTACAGCTCATCCATGCCC	This paper	N/A
Primer for cloning *UAS-Bnl:GFPΔFGF:* CCCTTGGACATGGACTGTGGCACCGTGG	This paper	N/A
Primer for cloning *UAS-Bnl:GFPΔFGF:* TGCCACAGTCCATGTCCAAGGGCGAGGAGC	This paper	N/A
Primer for cloning *UAS-Bnl:GFPΔFGF:* CACCGTCTTGTACAGCTCATCCATGCCC	This paper	N/A
Primer for cloning *UAS-Bnl:GFPΔFGF:* ATGAGCTGTACAAGACGGTGCCGCAGGAG	This paper	N/A
Forward primer for cloning all the constructs above: AATTCGAGCTCGGTACAGATCTATGCGAAGAAACCTGCGC	This paper	N/A
Primer for cloning *UAS-sBtl:Cherry*: AATTCGAGCTCGGTACCTCGAGATGGCAAAAGTGCCGATCACG	This paper	N/A
Primer for cloning *UAS-sBtl:Cherry*: GCCGCCTTGCCCCTCGACAGGATGGGCGTGCAGCAG	This paper	N/A
Primer for cloning *UAS-sBtl:Cherry*: GTCGAGGGGCAAGGCGGCatggtgagcaagggcgag	This paper	N/A
Primer for cloning *UAS-sBtl:Cherry*: GCCAAGCTTGCATGCCTCTAGAttacttgtacagctcgtccatgcc	This paper	N/A
Forward primer for *bnl* RT-PCR: CAGGAGGACACTCACAATTGCCAG	This paper	N/A
Reverse primer for *bnl-RA* RT-PCR: GCTGCAGACACAGGAAATCG	This paper	N/A
Reverse primer for *bnl-PC* RT-PCR: GGGACAACAGTCCGAAATCG	This paper	N/A
Primer for cloning *UAS-Bnl^{PC}:GFP:* GCCAAGCTTGCATGCCATATATTCTAGATCATCGCCGGGGGGACAACAGTCCGAAATCGTAGTAGAGCGAATCGTCG	This paper	N/A
Recombinant DNA		
pUAST-Bnl:GFP	[2]	N/A
pUAST-Bnl:HA	[2]	
pUAST-Bnl:HA_1GFP_3 (UAS-HA_1Bnl:GFP_3)	[2]	
pUAST-GFP-GPI	[7]	N/A
pUAST-cSpi:GFP	[8]	N/A
pUAST-Bnl:GFPΔC_{40} (ΔC) & -Bnl:GFPΔC_{168}	*UAS-Bnl:HA_1GFP_3* by deleting the last 40 (after Y_{730} of Bnl) and 168 (after K_{602} of Bnl) amino acid regions, respectively, prior to a stop codon.	N/A
pUAST-Bnl:GFPΔC-TM & -Bnl:GFPΔC-TM_{168}	a 31 amino acid long transmembrane domain of the mammalian CD8a protein fused to the C-terminus of *UAS-Bnl:GFPΔC* and *UAS-Bnl:GFPΔC_{168}*, respectively.	N/A

pUAST-Bnl:GFP$_3$Cherry$_c$	*UAS-Bnl:HA$_1$GFP$_3$* with a C-terminal mCherry tag with a linker (VEGQGG) placed in between.	N/A
pUAST-Bnl$_{PC}$:GFP	The PC-specific C-terminal 24 bp sequence (7 amino acids+stop) was added to the C-terminus of the 1-2259 bp region of *bnl-PA* CDS using PCR	N/A
pUAST-Bnl:GFP-ω^m	*UAS-Bnl:HA$_1$GFP$_3$* with mutated ω, ω+1, and ω+2 sites (S/P^{741}G/P^{742}A/P^{743})	N/A
pUAST-bGFP-GPI	secGFP [1] (superfolder GFP with N-terminal Bnl signal peptide) added with the last 53 amino acids of Bnl (from T$_{718}$) at the C-terminus.	N/A
pUAST-sBtl:Cherry	mCherry sequence was added in-frame after P^{607} of Btl, replacing the TM and intracellular portions.	N/A
pUAST-BtlDN:Cherry and pLot-BtlDN:Cherry	mCherry sequence was added in-frame after L$_{625}$ of Btl, replacing the intracellular C-terminal portions.	N/A
pUAST-Bnl:GFP$^{\Delta FGF}$	Conserved FGF domain of Bnl was replaced with a sfGFP sequence	N//A
Software and Algorithms		
Fiji	ImageJ	https://fiji.sc
Prism 8.0	GraphPad	https://www.graphpad.com/
Adobe Photoshop	Adobe	https://www.adobe.com
Adobe Illustrator	Adobe	https://www.adobe.com
Microsoft Excel	Microsoft	https://www.office.com
SnapGene	SnapGene	https://www.snapgene.com
MacVector	MacVector	https://macvector.com
PredGPI predictor		http://gpcr.biocomp.unibo.it/predgpi/pred.htm
VassarStats		vassarstats.net
R x64 3.3.1	R	r-project.org
Imaris 9.5.0	Imaris	https://imaris.oxinst.com

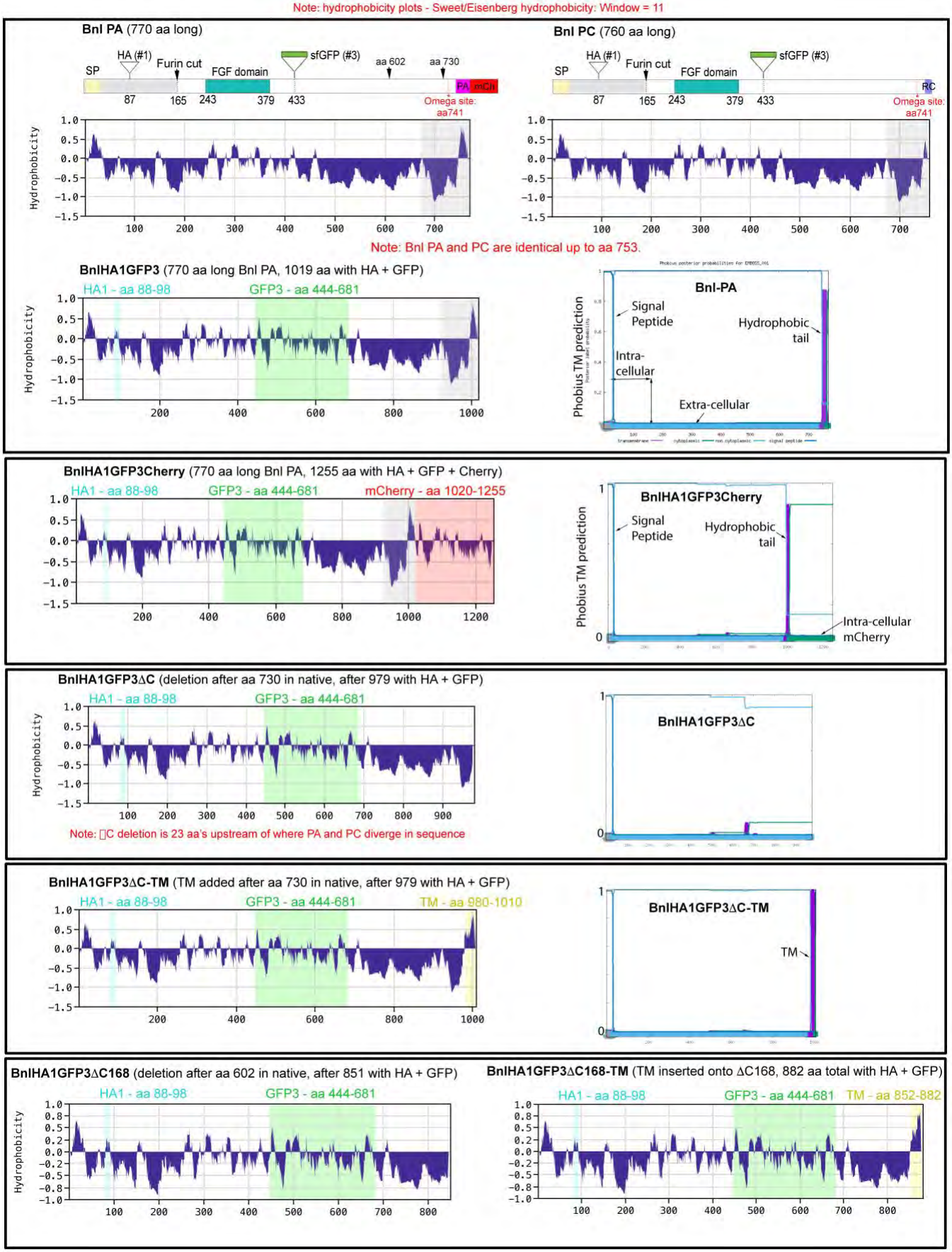
Supplementary Notes

A. Expression analyses of *bnl* splice variants using RT-PCR

The *bnl* gene has two different splice variants encoding proteins with different C-terminal hydrophobic sequences. To check the expression of different *bnl* isoforms, total RNA was extracted from 20 w^{1118} larval wing discs and RT-PCR was performed on the total RNA. For both isoforms, we used a common forward primer that binds to exon 3 (5'-CAGGAGGACACTCACAATTGCCAG-3'). However, the reverse primers were either PA- (5'-GCTGCAGACACAGGAAATCG-3') and PC- (5'-GGGACAACAGTCCGAAATCG-3') -specific. The reverse primer for each isoform was designed to span the junction between exon 3 and the isoform-specific last exon as illustrated below. The expected amplicon size was 697bp. As a negative control, RT-PCR was carried out on the genomic DNA (gDNA) template obtained from w^{1118} flies. As a positive control we performed RT-PCR for constitutive *rp49* gene. Unsurprisingly, RT-PCR results showed strong amplification of *bnl-PA,* exclusively from the RNA template. Although we detected strong *bnl-PC* amplification from the RNA template, we also detected a low level amplification of the same sized RT-PCR product from gDNA. These results confirmed *bnl-PA* expression in the wing disc. These results, although not conclusive, also suggested that the wing disc expresses *bnl-PC*. Moreover, a Bnl antibody, which detects both isoforms, showed that the native Bnl^{ex} is asymmetrically localized on the wing disc producing cell surface and the Bnl^{ex} was reduced with the PIPLC treatment. Secondly, S2 cells expressing a chimeric Bnl^{PC}:GFP construct showed the PIPLC-sensitive surface distribution of the protein. Based on these results, we suggest that irrespective of the tissue-specific expression levels, Bnl isoforms are GPI-anchored on the cell surface. Consistent RT-PCR results were obtained from three independent experiments, confirming the expression profile.



B. Bioinformatic analyses of hydropathy and secondary topology of various Bnl constructs



C. Comparison of *bnl-GAL4*-driven expression levels of transgenic constructs

Transgenic Constructs	Fly line	Chromosomal Insertion	Expression levels *	externalized by source?	ASP uptake?
UAS-Bnl:GFPΔC_{40}	2_2	3	+++	Yes	Yes
	3_1	3	ND	n/a	n/a
	3_2	2	++	Yes	Yes
UAS-Bnl:GFPΔC_{40}-TM	1_2	3	+++	Yes	Yes (R)
	2_2	2	+++	Yes	Yes (R)
	3_1	3	++	Yes	Yes (R)
	3_2	2	++	Yes	Yes (R)
	4_1	2	++	Yes	Yes (R)
	6_1	3	ND	n/a	n/a
	7_1	3	+	Yes	Yes (R)
	9_2	3	+++	Yes	Yes (R)
UAS-Bnl:GFPΔC_{168}	1_2	2	++	Yes	Yes
	4_1	3	+++	Yes	Yes
UAS-Bnl:GFPΔC_{168}-TM	1_1	3	++	Yes	Yes (R)
	2_1	3	+++	Yes	Yes (R)
UAS-Bnl:GFP-ω^m	1_1	3	+++	L	L
	4_1	2	+++	L	L

* Expression levels were verified by *bnl-Gal4* driven expression of the *UAS* constructs in the wing disc *bnl* source (see Methods). Bnl:GFP lines used in this study was published earlier in Du et al. and Sohr et al.[1,2]. ND: Not detected. Red: Lines with comparable expression levels used in this study. R: Restricted range. L: Very low level.

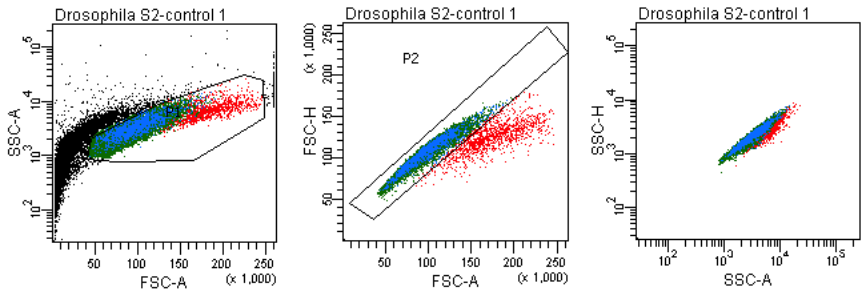
+++ >++>+ : High>medium>low levels of expression relative to each other.

An example of comparison of levels of expression of Bnl:GFP variants under *bnl-Gal4* in the wing disc

	Bnl:GFP	Bnl:GFPΔC_{40} (2_2)	Bnl:GFPΔC_{40}-TM (2_2)
Mean GFP intensity in the *bnl*-source (derived from extended Z-stack of 50 μm tissue) (7 wing discs from 7 animals used)	1137.40	867.5	1495.3
	1142.2	1106.3	899.7
	1870.1	2400.1	620.6
	747.7	909.8	1372.5
	1190.05	782.9	610.1
	972.8	985.5	1868.6
	527.6	484.3	1126.298
Average	**1083.98***	**1076.63***	**1141.87***

*, Comparable levels of expression of different constructs used in this study.

D. Examples of gating strategy for FACS analyses



Supplementary References

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2. Sohr, A., Du, L., Wang, R., Lin, L. & Roy, S. Drosophila FGF cleavage is required for efficient intracellular sorting and intercellular dispersal. *J Cell Biol* 218, 1653–1669 (2019).

3. Roy, S., Huang, H., Liu, S. & Kornberg, T. B. Cytoneme-mediated contact-dependent transport of the Drosophila decapentaplegic signaling protein. *Science (New York, N.Y.)* 343, 1244624 (2014).

4. Reichman-Fried, M. & Shilo, B.-Z. Breathless, a Drosophila FGF receptor homolog, is required for the onset of tracheal cell migration and tracheole formation. *Mech Develop* 52, 265–273 (1995).

5. Sato, M. & Kornberg, T. B. FGF Is an Essential Mitogen and Chemoattractant for the Air Sacs of the Drosophila Tracheal System. *Dev Cell* 3, 195–207 (2002).

6. Cabernard, C. & Affolter, M. Distinct Roles for Two Receptor Tyrosine Kinases in Epithelial Branching Morphogenesis in Drosophila. *Dev Cell* 9, 831–842 (2005).

7. Greco, V., Hannus, M. & Eaton, S. Argosomes: a potential vehicle for the spread of morphogens through epithelia. *Cell* 106, 633–645 (2001).

8. Miura, G. I. *et al.* Palmitoylation of the EGFR ligand Spitz by Rasp increases Spitz activity by restricting its diffusion. *Developmental Cell* 10, 167–176 (2006).

9. Greco, V., Hannus, M. & Eaton, S. Argosomes: a potential vehicle for the spread of morphogens through epithelia. *Cell* 106, 633–645 (2001).

R plot code

```
library(ggplot2)

filename="Control Source"

file_t= "data\\"
file<-paste(file_t,filename,".csv",sep="")

#data<-read.csv("FinalData\\Control Source.csv",head=TRUE,sep=",")
data<-read.csv(file,head=TRUE,sep=",")

cols<-c("#619cff","#f8766d","#00ba38")
#cols<-c("red","blue","green")

p<-ggplot(data, aes(x=data$Range,y=data$Counts,fill=data$Length)) +
 #theme_bw()+
 #theme_minimal() +
 geom_bar(width = 30, colour="black", stat="identity") +
 #geom_hline(yintercept = 2.5) +
 #geom_vline(xintercept = c(0,90,180,270)) +
 scale_fill_manual(values = cols) +
 #scale_y_discrete(drop = FALSE) +
 theme(legend.box.just = "top",legend.position = "bottom") +
 theme(panel.grid.major = element_line(colour = "gray"),
        panel.grid.minor = element_line(colour = "blue"),
        panel.background = element_blank(),
        axis.line = element_line(colour = "black"))+
 labs(title = filename,
        fill = "Length range(um)",
        y = "Cytoneme number", limits = c(0, 100),colour =
"Cylinders") +
 coord_polar(theta = "x", start=pi/2, direction=-1) +
 scale_x_discrete("",  limits = c(0,90,180,270), labels =
c(0,90,180,270)) +
 scale_y_continuous(limits=c(0,5), breaks= c(1:5))


p
```