Godzilla-dependent transcytosis promotes Wingless signalling in *Drosophila* wing imaginal discs

Yasuo Yamazaki, Lucy Palmer, Cyrille Alexandre, Satoshi Kakugawa, Karen Beckett, Isabelle Gaugue, Ruth H. Palmer and Jean-Paul Vincent

The apical and basolateral membranes of epithelia are insulated from each other, preventing the transfer of extracellular proteins from one side to the other. Thus, a signalling protein produced apically is not expected to reach basolateral receptors. Evidence suggests that Wingless, the main *Drosophila* Wnt, is secreted apically in the embryonic epidermis. However, in the wing imaginal disc epithelium, Wingless is mostly seen on the basolateral membrane where it spreads from secreting to receiving cells. Here we examine the apico-basal movement of Wingless in Wingless-producing cells of wing imaginal discs. We find that it is presented first on the apical surface before making its way to the basolateral surface, where it is released and allowed to interact with signalling receptors. We show that Wingless transcytosis involves dynamin-dependent endocytosis from the apical surface. Subsequent trafficking from early apical endosomes to the basolateral surface requires Godzilla, a member of the RNF family of membrane-anchored E3 ubiquitin ligases. Without such transport, Wingless signalling is strongly reduced in this tissue.

Two immunofluorescence staining protocols were used to examine the apico-basal distribution of Wingless in wing imaginal discs. The standard protocol, which includes a permeabilization step, is expected to show intracellular and extracellular protein. Owing to the relatively higher abundance of Wingless in the intracellular pool, this method preferentially highlights intracellular Wingless. With this protocol, Wingless was seen to accumulate largely in the apical region (Fig. 1ad). To detect extracellular Wingless, imaginal discs were stained and fixed in the absence of detergent. With this staining protocol, Wingless was seen mostly along the basolateral surface of producing and nearby receiving cells (Fig. 1i–l). Although Wingless was enriched along the basal half, some signal could be detected up to the level of adherens junctions, recognized with anti-E-cadherin (Supplementary Fig. 1a). Overall, these observations suggest that Wingless protein is produced in the apical region and that somehow it is transported to the basolateral surface to be released in the extracellular space.

To confirm that Wingless is indeed first produced apically before proceeding to the basolateral surface, we devised a method to track it in the secretory pathway and beyond. Two *wingless* complementary DNAs, each carrying a different epitope tag, were inserted in tandem at the *wingless* locus in such a way that no endogenous Wingless activity remained, and only the first cDNA (OLLAS-tagged) was expressed (Fig. 2a). This first cDNA was flanked by Flp recombination targets (FRT sites, denoted as F in the genotypes below) allowing its excision on FLP expression. As excision also removes the transcriptional termination signal associated with the first cDNA, it is expected to trigger expression of the second cDNA (HA-tagged). We refer to this genotype as *wg*KO(F–*Wg*OLLAS–*F–Wg*HA). Neither tag affected protein functionality, as indicated by the fact that homozygous *wg*KO(F–*Wg*OLLAS–*F–Wg*HA) flies (Supplementary Fig. 1b), as well as homozygous *wg*KO(Wg*HA) flies, are fully viable, with a normal wing margin. As the wing margin is particularly sensitive to reduced Wingless signalling, these observations confirm that both WinglessOLLAS and WinglessHA have full signalling activity. Expression of FLP throughout the posterior compartment (under the control of *hedgehog-*Gal4) led to complete conversion from one tag to another (Supplementary Fig. 1c), showing that the *F–Wg*OLLAS–*F* cassette is readily excised. To make conversion inducible, a *tubulin–Gal80* transgene was introduced (Fig. 2a). At 18°C, Gal80 is

---

1Department of Medical Biochemistry and Cell Biology, Institute of Biomedicine, The Sahlgrenska Academy at the University of Gothenburg, Medicinaregatan 9A, 40539 Gothenburg, Sweden. 2The Francis Crick Institute, Mill Hill Laboratory, The Ridgeway, Mill Hill, London NW7 1AA, UK. 3Polarity, Division and Morphogenesis Team, Institut Curie, CNRS UMR 3215, INSERM U934, 26 rue d’Ulm, 75248 Paris Cedex 05, France. 4These authors contributed equally to this work. 5These authors jointly supervised this work. 6Correspondence should be addressed to R.H.P. or J.-P.V. (e-mail: ruth.palmer@gu.se or jp.vincent@crick.ac.uk)

Received 30 September 2015; accepted 5 February 2016; published online 14 March 2016; DOI: 10.1038/ncb3325
active, preventing Gal4 from triggering FLP expression. Shifting to 29°C inactivates Gal80, inducing FLP expression and hence causing expression from the wingless locus to switch from WinglessKO;GAL80 to WinglessHA. The switch occurred with a delay, as expected, and with some cell-to-cell heterogeneity. Nevertheless, a clear temporal sequence could be seen. The first signs of HA immunoreactivity in permeabilized discs appeared 2 h after the temperature shift. At this time, 95% of all HA-positive punctae were found in the apical half (n = 55) and most of them (87%) overlapped with a Golgi marker (anti-GMAP; Fig. 2d). This confirms that Wingless is translated and processed in apically located secretory compartments. From 3 h after the temperature shift, the number of HA-positive, GMAP-negative punctae decreased to 42% (n = 97), most probably corresponding to late secretory vesicles or early endosomes (Fig. 2e). Beyond this time (for example, 8 h after the temperature shift), HA immunoreactivity was seen to spread in a basal direction (Fig. 2f). From about 16 h after the temperature shift, HA immunoreactivity was seen along the whole apico-basal axis of every HA-positive cell located within the Wingless-expressing domain (12 cells from 3 discs analysed), as is the case for wild-type Wingless at the steady state. These cells were flanked by HA signal seemingly associated with non-expressing cells (arrows in Fig. 2f), perhaps representing Wingless released from the basolateral surface. The above observations support the notion that Wingless undergoes apical-to-basal transport. A paracellular trans epithelial route is highly unlikely because there is no evidence that Wingless could reorganize junctional components. Instead, Wingless could either traffic from an apical Golgi compartment directly to the basolateral surface or travel first to the extracellular apical surface before undergoing apico-basal transcytosis.

If Wingless uses the apical surface as a stopover before being endocytosed and trafficked to the basolateral surface, then acute inhibition of endocytosis should lead to apical accumulation of extracellular Wingless. In flies hemizygous for the shibire allele, all dynamin-dependent endocytosis arrests within a few minutes after transfer from the permissive temperature of 18°C to the restrictive temperature of 34°C (refs 8,9). The distribution of extracellular Wingless was examined in imaginal discs from shibire/Y larvae cultured for 15, 30 and 60 min at 34°C. Within 15 min, Wingless was seen to accumulate at the apical surface and this was followed by depletion from the basolateral surface (Fig. 2k–n and Supplementary Fig. 1d–g). Note that such depletion is expected if supply to the basolateral surface (for example, from the apical surface) is cut off while Wingless continues to be lost at the basolateral surface (for example, through release). The shibire mutation is completely reversible, a feature that allowed us to track the fate of accumulated apical Wingless on restoration of dynamin activity following return to 18°C. After 30 min at this temperature, extracellular apical Wingless reverted to its low, steady-state level. Within a 90 min period, basolateral Wingless was replenished to the levels seen before the endocytosis block (Fig. 2o–r and Supplementary Fig. 1h–k). These results are consistent with the scenario that, in the wild type, Wingless first reaches the apical surface before progressing to the basolateral surface. We suggest that it is from there that Wingless can reach adjoining cells. Further Wingless spread, which has been shown to take place, even if it is not absolutely essential, is likely to be initiated from the basolateral surface too.

Several membrane-associated proteins and trafficking components could be involved in Wingless transcytosis. Glycans have been

**Figure 1** Apical accumulation of intracellular Wingless and basolateral enrichment of extracellular Wingless. (a–d) Third instar wing imaginal discs stained with anti-Wingless in the presence of detergent. Shown are the whole pouch (a), a magnified view (b), a cross-section (c) and a longitudinal section (d) of the expression stripe. Note the relative accumulation of Wingless in the apical region. (e–h) Similar views of an imaginal disc processed for in situ hybridization with a wingless probe. Most of the signal is also enriched in the apical region. (i–l) Similar views of an imaginal disc stained to show extracellular Wingless. Here, the signal is much weaker than in a–d (not shown at the same gain). Most of the signal is basolateral. A grey dashed line in c, g and k marks the approximate plane of focus of the images shown in a/b, e/f and i/j, respectively. Apical is shown at the top in this and all subsequent figures. Every picture is representative of data from >10 discs from three independent experiments. Scale bars, 24 μm.
shown to undergo transcytosis\textsuperscript{12} and it has been suggested from the results of overexpression experiments that the glypican Dally-like (Dlp) could ferry Wingless across the epithelium\textsuperscript{13}. To test the requirement of glypicans, we removed Dlp as well as Dally, the only (Dlp) could ferry Wingless across the epithelium

Figure 2 Tracking the progress of Wingless along the apico-basal axis. (a) Diagram depicting the tag switching construct inserted at the \textit{wingless} locus. (b) Steady-state distribution of endogenous Wingless relative to the Golgi marker GMAP in a third instar \textit{wing} imaginal disc. 82\% of Wingless-positive punctae are in the apical half (\textit{n} = 175). (c) Absence of HA immunoreactivity in an imaginal disc from \textit{wg}\textsuperscript{F–Wg\textsuperscript{OLLAS–F–Wg\textsuperscript{OLLAS}}/UAS–Flp; hedgehog–Gal4 tub–Gal80\textsuperscript{°}} larva cultured at 18 °C. (d) HA immunoreactivity can be seen 2 h after transfer to 29 °C and mostly co-localizes with GMAP (white arrows). (e) From 3 h onwards, anti-HA is found in both GMAP-negative (blue arrows) as well as GMAP-positive (white arrows) vesicles. (f) Steady-state distribution of Wingless\textsuperscript{OLLAS} in a \textit{Wg\textsuperscript{OLLAS–F–Wg\textsuperscript{OLLAS}}/UAS–Flp; hedgehog–Gal4 tub–Gal80\textsuperscript{°}} imaginal disc. Note the absence of Wingless\textsuperscript{HA}. (g,h) HA-immunoreactive vesicles first appear in the apical region 2 h after the temperature shift (g), and increase in number during the following 2 h (h). (i) Wingless\textsuperscript{HA} is seen at even more basolateral positions 8 h after transfer to 29 °C. (j) Another 8 h later, Wingless\textsuperscript{HA} has completely replaced Wingless\textsuperscript{OLLAS} and HA immunoreactivity can also be detected in nearby non-expressing cells (white arrows). Each panel from b to j shows merged (left) and individual (right) channels. (k) This and all subsequent panels show hemizygous \textit{shi\textsuperscript{16}} \textit{wing} imaginal discs at the third instar. When endocytosis is allowed to proceed normally (18 °C), steady-state extracellular Wingless is mainly found at the basolateral surface (approximate plane of focus is shown as a grey line across a diagrammatic cell on the left). (l–n) Preventing dynamin-dependent endocytosis leads to Wingless accumulation at the apical surface and concomitant depletion from the basolateral surface (15, 30 and 60 min at 34 °C). (o) Steady-state extracellular Wingless with normal endocytosis (18 °C). (p) Extracellular Wingless after endocytosis block (60 min at 34 °C). (q,r) Restoration of dynamin activity by transfer to 18 °C for 30 min (q) and 90 min (r). This leads to removal of accumulated apical extracellular Wingless and replenishment of the basolateral pool. Data from b–j, that is, the number of punctae, as provided in the text, were pooled from at least three discs per experimental condition. Each time point in k–n is representative of >10 discs from three independent experiments. For o–r, >5 discs from three independent experiments were analysed. Scale bars, 20 μm (b–j) and 24 μm (k–r).
Figure 3 Loss of Godzilla or Synaptobrevin interferes with Wingless signalling and trafficking. (a,b) Depletion of godzilla by RNAi in the posterior compartment (hedgehog–Gal4; UAS–gzlRNAi) results in loss of margin in adult wings (arrowhead in b). (c,d) Expression of the godzilla RNAi transgene in the posterior compartment of third instar imaginal discs (marked by co-expression of GFP and indicated by a blue line) causes loss of Senseless expression (c,d) and Wingless accumulation (c,d'). The GFP-negative compartment serves as a control. Note that residual Senseless remains in the affected territory, in a variable fashion. Panels in e show a subapical plane and d an optical transverse section. Pictures are representative of >10 discs. (e,f) Accumulation of Wingless protein in godzillaΔ2 (gzlΔ2) clones marked by the absence of GFP. Panels in e show a subapical plane and f an optical transverse section. (g) Adult wings bearing unmarked syb144 mutant clones have notches in the margin (arrowhead). 30% of examined discs had a similar phenotype. (h) Expression of an RNAi transgene against syb in the posterior compartment driven, along with GFP, by hedgehog–Gal4 leads to loss of Senseless expression. (i,j) Wingless protein accumulates in Wingless-producing cells that are homozygous for syb144 (random clones induced in the posterior compartment, marked by absence of GFP). Panels in i show a subapical plane and j an optical transverse section. All of the pictures are representative of >10 samples (wings or discs) from at least three independent experiments. Scale bars represent 10 μm, except in a,b and g, where they represent 500 μm.

surface (Supplementary Fig. 2). Another membrane-associated protein that could conceivably contribute to Wingless transcytosis is the multi-pass transmembrane protein encoded by wntless/evenness interrupted/sprinter, here referred to as Evi (refs 15–17). Evi and its mammalian homologues are known to interact and traffic with Wingless/Wnts18,19 and AP-2-dependent Evi recycling has been suggested to contribute to basolateral secretion of Wnt3A in MDCK cells20. However, the roles of AP-2 and Evi in transcytosis are hard to test because AP-2 is required in many other processes and because Evi is needed for Wingless to reach the cell surface15,21.

Besides requiring a membrane-associated escort, Wingless transcytosis is likely to involve trafficking regulators, for example to ensure appropriate sorting out of early endosomes. From RNA-mediated interference (RNAi)-based analysis of various candidates, we found that knocking down godzilla, which encodes a PA–TM–RING E3 ubiquitin ligase that regulates recycling endosome trafficking22, led to loss of wing margin (Fig. 3a,b), a structure that requires Wingless signalling at the third instar7. Accordingly, godzilla knockdown (confirmed by immunostaining; Supplementary Fig. 3a,b) led to a strong reduction in the expression of Senseless (Fig. 3c,d), a target of Wingless signalling in late wing imaginal discs23. Note that expression of a wingless–lacZ enhancer trap was largely unaffected in this context (Supplementary Fig. 3c), indicating that Notch signalling, which is required for wingless transcription at the prospective margin24, is relatively unaffected by godzilla knockdown. However, following godzilla knockdown, the Wingless protein distribution was noticeably restricted, as if confined to the expression domain (Fig. 3c). Moreover, godzilla knockdown caused accumulation of intracellular Wingless in Wingless-expressing cells, particularly in the apical region (Fig. 3d). To confirm the specificity of this phenotype, we generated homozygous godzillaΔ2 clones. Unfortunately, large clones could not be recovered, perhaps because they are outcompeted. However, intracellular Wingless was seen to accumulate in the small clones that were generated (Fig. 3e,f). These observations are consistent with the notion that Godzilla normally helps Wingless to exit early apical endosomes. Previous work has shown that Godzilla regulates
endocytic trafficking by ubiquitinating VAMP3, a SNARE protein also known as Synaptobrevin (Syb) in Drosophila\textsuperscript{22}. We therefore asked whether the role of Godzilla in Wingless trafficking could be mediated by Syb. Induction of syb mutant clones (unmarked) caused wing margin defects and syb knockdown throughout the posterior compartment led to loss of Senseless expression, suggesting a role for Syb in Wingless signalling (Fig. 3g,h). The wingless-\textasciitilde{lacZ} reporter was unaffected by Syb knockdown (Supplementary Fig. 3d), suggesting that, as with godzilla knockdown, wingless transcription is maintained under these experimental conditions. Critically, Wingless was seen to accumulate in syb mutant clones (Fig. 3i,j), the same phenotype as that seen in Godzilla defective cells. The above results suggest that Godzilla, and its target Syb, regulate a post-endocytic event that ensures Wingless transcytosis. Accordingly, Wingless and Godzilla are expected to transit through common Rab5-positive endosomes. Indeed, Wingless and Godzilla were found to co-localize in enlarged endosomes induced by expression of YFP-tagged constitutively active Rab5 (Supplementary Fig. 4a,b). Likewise, overexpression of Godzilla caused the formation of enlarged endosomes where Wingless accumulates (Supplementary Fig. 4c). It is conceivable therefore that Godzilla could meet Wingless in early endosomes and direct it towards an onward transcytotic route.

To test the contribution of Godzilla to Wingless transcytosis, the distribution of extracellular Wingless was assessed following Godzilla knockdown. As shown in Fig. 4a–c, this led to apical accumulation and basolateral depletion, the same phenotype seen on acute inhibition of endocytosis in shibire\textsuperscript{22}/\textasciitilde{Y} imaginal discs. To confirm this observation, we designed a dominant-negative version of Godzilla (Gzl.LD) by mutating key residues (His255 and His258) in the RING domain (Fig. 4d). Expression of this construct led to the same phenotype as that caused by RNAi-mediated knockdown (Fig. 4e–g). Also consistent with the results of RNAi knockdown, this was accompanied by impaired signalling, as indicated by wing margin defects and loss of Senseless expression (Supplementary Fig. 4d,e). We suggest therefore that Godzilla could control a switch that directs cargo (for example, Wingless) from early endosomes into a transcytotic route. Analysing the role of Godzilla in the behaviour of other transcytosed cargoes will be needed to determine whether Godzilla is a general controller of apical-to-basolateral transcytosis, as suggested by the requirement of Godzilla for long-term cell viability.

Why does Wingless need to undergo transcytosis? The need for trafficking to the basolateral surface could be explained if Wingless was preferentially released and/or mostly signalled there. We therefore devised an independent experimental test of whether Wingless normally signals from the apical or the basolateral surface. This test relies on the fact that the haemolymph (Drosophila blood) has access only to the basolateral surface of imaginal discs. Topologically, the apical surface faces the outside of the larva. We used a Gal4 driver active in the fat body and haemocytes (cg-Gal4) to flood the haemolymph with Notum, an enzyme that inactivates Wingless in the extracellular space by removal of its palmitoleate moiety\textsuperscript{25}. As sustained systemic expression of Notum was lethal, Gal80\textasciitilde{ts} was used to induce expression for a limited time. Fifteen hours was found to be compatible with larval viability. During this time, Notum accumulated at imaginal discs and, as a result, expression of Senseless was inhibited, although to varying degrees, most probably depending on the extent of Notum expression.

**Figure 4** Knockdown of godzilla prevents Wingless transcytosis. (a,b) En face views of a third instar wing imaginal disc expressing RNAi against godzilla (marked by GFP co-expression) in the posterior compartment (with engrailed–Gal4). Staining for extracellular Wingless shows that godzilla RNAI causes Wingless to accumulate at the apical surface (a) and to become depleted from the basolateral surface (b). (c) The same preparation shown in a transverse optical section. This disc is representative of >10 discs from two independent experiments. (d) Diagram of Godzilla indicating the two conserved residues (His 255 and His 258) that were mutated to generate a ligase-dead version. (e–g) Expression of GFP-tagged ligase-dead Godzilla (Gzl.LD,GFP) phenocopies godzilla knockdown: accumulation of extracellular Wingless at the apical surface (e) and depletion at the basolateral surface (f). Panel g shows a transverse optical section. This disc is representative of >10 discs from three independent experiments. Scale bars, 10 μm.
in the haemolymph (Fig. 5a,b). We cannot exclude the possibility that some Notum could reach the apical surface in this experimental setup. However, we think it is unlikely because wing imaginal discs have been shown to form a tight subapical seal26. We conclude therefore that Wingless is unlikely to trigger sufficient signalling (at least to a level that triggers Senseless expression) during its brief transit at the apical surface. Wingless signal transduction is mediated by Arrow (LRP5/6) and the redundant Frizzled and Frizzled2. To further investigate the likely location of signal transduction, we sought to determine the subcellular localization of Frizzled2. As no suitable antibodies were available, we modified the frizzled2 locus such that the product would be tagged with GFP. In situ hybridization has shown that frizzled2 expression is repressed by Wingless signalling27. This was confirmed by the distribution of GFP–Frizzled2, low near the site of Wingless expression and relatively higher further away (Fig. 5c and Supplementary Fig. 5). Importantly, GFP (hence, Frizzled2) was predominantly found at the basal surface (Fig. 5c). We suggest that this could account, at least in part, for the inability of Wingless to trigger significant signalling activity at the apical surface.

The experimental disc shown is representative of nine discs from six larvae. (c) Distribution of GFP-tagged Frizzled2 (detected with anti-GFP) in third instar imaginal discs. The yellow line indicates the position of the optical sections shown below the (peripodial membrane is cropped out for clarity). Most of the staining is found at the basal surface, as shown in the transverse optical section. Note that Frizzled2 is relatively depleted around the source of Wingless, as expected because frizzled2 is transcriptionally repressed by Wingless signalling27. Scale bars, 24 μm (a-c). (d) A model depicting the role of Godzilla in Wingless transcytosis. Godzilla (represented by the cartoon Godzilla monster) normally contributes to exit from early endosomes, thus ensuring that Wingless reaches the basolateral surface where it engages with signalling receptors to trigger target gene expression.

The question remains as to why Wingless needs to transit through the apical surface before reaching the basolateral surface. Several possibilities can be considered. Wingless could somehow require transcytosis to become active, perhaps as a result of the pH changes it experiences in endocytic compartments. Indeed, acidification in the secretory pathway has been suggested to stimulate Wnt release18. Alternatively, Wingless’s circuitous route out of the cell could be necessary to prevent interaction in the secretory pathway with Notum, which is expected to be co-expressed because it is a target gene25.

In conclusion, we have shown that Wingless is first presented at the apical surface of wing imaginal disc cells before being transcytosed to the basolateral surface, where it triggers signalling (Fig. 5d). These results build on earlier evidence that intracellular trafficking modulates Wingless signalling23. Transcytosis of Wnt3a has been shown to take place in cultured mammalian cells20, and Hedgehog, another lipid-modified signalling protein, also undergoes transepithelial transcytosis26, suggesting that it could be a general process. Further work will be needed to determine whether vertebrate Wnt transcytosis occurs in vivo and also whether Wingless transcytosis

Figure 5 Basolateral Notum originating from the haemolymph inhibits Wingless signalling in wing imaginal disc. (a,a’) Control imaginal disc carrying UAS–Notum and tubulin–Gal80 (no cg-gal4). No V5 immunoreactivity is detected (a) and expression of Senseless is normal (a’). The main panels show en face views and the small rectangles show X–Z reconstructions at the level of the Wingless stripe. (b,b’) Experimental disc carrying UAS–Notum, tubulin–Gal80 and cg-Gal4 (fat body and haemocyte driver). Control and experimental discs were co-cultured first at 18 °C to allow growth and survival and then at 29 °C for 15 h to inactivate Gal80, hence triggering Notum expression into the haemolymph of experimental animals. This led to accumulation of Notum–V5 (b) and loss of Senseless expression (b’). These effects were variable, most probably because of suboptimal Notum expression (longer time at 29 °C caused larval lethality).
takes place in other epithelial tissues of Drosophila, such as in the embryonic epidermis. As we have shown, Godzila, the Drosophila homologue of RNF167, a membrane-associated E3 ubiquitin ligase, promotes transcytosis of Wingless and possibly other cargoes. This activity is distinct from that of another membrane-associated ubiquitin ligase RNF43, which triggers the removal of Wnt receptors from the cell surface\(^5\). It is also distinct from the activity of RNF146, which regulates the degradation of Axin, a component of the β-catenin destruction complex\(^6\). Thus, RNF43 and RNF146 seem to be specific regulators of Wnt signalling. In contrast, Godzila and its mammalian counterpart (RNF167) seem to regulate a more pleiotropic process, that is, transcytosis.

**METHODS**

Methods and any associated references are available in the online version of the paper.

**ACKNOWLEDGEMENTS**

This work was supported by the MRC (U117584268 to J.-P.V.), the European Union (ERC grant WNTEXPORT; 294523 to J.-P.V.), the Swedish Cancer Society (15:391 to R.H.P.), the Swedish Children Cancer Foundation (2015-04466 to R.H.P.), and the SSF Programme Grant to R.H.P., the Swedish Research Council (2015-04466 to R.H.P.), and the Swedish Children Cancer Foundation (2015-0096 to R.H.P.), the European Union requires Evi, a conserved transmembrane protein. Cell 125, 523–533 (2006).

16. Bartscherer, K., Pelete, N., Ingelfinger, D. & Boutros, M. Secretion of Wnt ligands requires Evi, a conserved transmembrane protein. Cell 125, 523–533 (2006).

17. Goodman, R. M. et al. Sprintr: a novel transmembrane protein required for Wg secretion and signaling. Development 133, 4901–4911 (2006).

18. Combs, G. S. WLS-dependent secretion of WNT3A requires Ser209 acylation and vacuolar acidification. J. Cell Sci. 123, 3357–3367 (2010).

19. Herr, P. & Basler, K. Porcupine-mediated lipidation is required for Wnt recognition by Wls. Dev. Biol. 361, 392–402 (2012).

20. Yamamoto, H. et al. The apical and basolateral secretion of Wnt11 and Wnt3a in polarized epithelial cells is regulated by different mechanisms. J. Cell Sci. 126, 2931–2943 (2013).

21. Yu, J. et al. WLS retrograde transport to the endoplasmic reticulum during Wnt secretion. Dev. Cell 29, 277–291 (2014).

22. Yamazaki, Y. et al. Goliath family E3 ligases regulate the recycling endosome pathway via VAMP3 ubiquitylation. EMBO J. 32, 524–537 (2013).

23. Seto, E. S. & Bellien, H. J. Internalization is required for proper Wingless signaling in Drosophila melanogaster. J. Cell Biol. 173, 95–106 (2006).

24. Ruhlson, E. J. & Blair, S. S. Notch regulates wingless expression and is not required for reception of the paracrine wingless signal during wing margin neurogenesis in Drosophila. Development 121, 2813–2824 (1995).

25. Nakamura, Y. et al. Notum deacetylase Wnts suppressing signalling activity. Nature 519, 187–192 (2015).

26. Oshima, K. & Fehon, R. G. Analysis of protein dynamics within the septate junction defines a highly stable core protein complex that does not include the basolateral counterpart (RNF167) seem to regulate a more pleiotropic process, that is, transcytosis.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

Published online at http://dx.doi.org/10.1038/ncb3325

Reprints and permissions information is available online at www.nature.com/reprints

1. Tuma, P. L. & Hubbard, A. L. Transcytosis: crossing cellular barriers. Physiol. Rev. 83, 871–932 (2003).
2. Willkie, G. S. & Davis, I. Drosophila wingless and pair-rule transcripts localize apically by dynein-mediated transport of RNA particles. Cell 105, 209–219 (2001).
3. Simmonds, A. J., dos Santos, G., Lione-Bar, I. & Krause, H. M. Apical localization of wingless transcripts is required for wingless signaling. Cell 105, 197–207 (2001).
4. Strigini, M. & Cohen, S. M. Wingless gradient formation in the Drosophila wing. Curr. Biol. 10, 293–300 (2000).
5. Wu, J., Klein, T. J. & Mlodzik, M. Subcellular localization of frizzled receptors, mediated by their cytoplasmic tails, regulates signaling pathway specificity. PLoS Biol. 2, E158 (2004).
6. Baena-Lopez, L. A., Alexandre, C., Mitchell, A., Pasakarnis, L. & Vincent, J. P. Accelerated homologous recombination and subsequent genome modification in Drosophila. Development 140, 4818–4825 (2013).
7. Couse, J. P., Bishop, S. A. & Martinez Arias, A. The wingless signalling pathway and the patterning of the wing margin in Drosophila. Development 120, 621–636 (1994).
8. Van der Bliek, A. M. & Meyerowitz, E. M. Dynamin-like protein encoded by the Drosophila shibire gene associated with vesicular traffic. Nature 351, 411–414 (1991).
9. Kicheva, A. et al. Kinetics of morphogen gradient formation. Science 315, 521–525 (2007).
10. Zecca, M., Basler, K. & Struhl, G. Direct and long-range action of a wingless morphogen gradient. Cell 87, 833–844 (1996).
11. Alexandre, C., Baena-Lopez, A. & Vincent, J.-P. Patterning and growth control by membrane-tethered Wingless. Nature 505, 180–185 (2014).
12. Mertens, G., Van der Schueren, B., van den Berghe, H. & David, G. Heparan sulfate expression in polarized epithelial cells: the apical sorting of glypicans (GPI-anchored proteoglycan) is inversely related to its heparan sulfate content. J. Cell Biol. 132, 487–497 (1996).
13. Gallet, A., Staccini-Lavenant, L. & Thérond, P. P. Cellular trafficking of the glypican Dally-like is required for full-strength Hedgehog signaling and wingless transcytosis. Dev. Cell 14, 712–725 (2008).
14. Han, C., Yan, D., Beledkaya, T. Y. & Lin, X. Drosophila glypicans Dally and Dally-like shape the extracellular Wingless morphogen gradient in the wing disc. Development 132, 667–679 (2005).
15. Bänziger, C. et al. Wntless, a conserved membrane protein dedicated to the secretion of Wnt proteins from signaling cells. Cell 125, 509–522 (2006).
16. Bartscherer, K., Pelete, N., Ingelfinger, D. & Boutros, M. Secretion of Wnt ligands requires Evi, a conserved transmembrane protein. Cell 125, 523–533 (2006).
17. Goodman, R. M. et al. Sprintr: a novel transmembrane protein required for Wg secretion and signaling. Development 133, 4901–4911 (2006).
18. Combs, G. S. WLS-dependent secretion of WNT3A requires Ser209 acylation and vacuolar acidification. J. Cell Sci. 123, 3357–3367 (2010).
19. Herr, P. & Basler, K. Porcupine-mediated lipidation is required for Wnt recognition by Wls. Dev. Biol. 361, 392–402 (2012).
20. Yamamoto, H. et al. The apical and basolateral secretion of Wnt11 and Wnt3a in polarized epithelial cells is regulated by different mechanisms. J. Cell Sci. 126, 2931–2943 (2013).
21. Yu, J. et al. WLS retrograde transport to the endoplasmic reticulum during Wnt secretion. Dev. Cell 29, 277–291 (2014).
22. Yamazaki, Y. et al. Goliath family E3 ligases regulate the recycling endosome pathway via VAMP3 ubiquitylation. EMBO J. 32, 524–537 (2013).
23. Seto, E. S. & Bellien, H. J. Internalization is required for proper Wingless signaling in Drosophila melanogaster. J. Cell Biol. 173, 95–106 (2006).
24. Ruhlson, E. J. & Blair, S. S. Notch regulates wingless expression and is not required for reception of the paracrine wingless signal during wing margin neurogenesis in Drosophila. Development 121, 2813–2824 (1995).
25. Nakamura, Y. et al. Notum deacetylase Wnts suppressing signalling activity. Nature 519, 187–192 (2015).
26. Oshima, K. & Fehon, R. G. Analysis of protein dynamics within the septate junction defines a highly stable core protein complex that does not include the basolateral counterpart (RNF167) seem to regulate a more pleiotropic process, that is, transcytosis.

NATURE CELL BIOLOGY VOLUME 18 | NUMBER 4 | APRIL 2016

© 2016 Macmillan Publishers Limited. All rights reserved
METHODS

Immunostaining and microscopy. The following primary antibodies were used: mouse anti-Wingless (1:1,000 for standard protocol and 1:300 for extracellular staining; DSHB 4D4), rabbit anti-HA (1:1,000, Cell Signaling), rat anti-OLLAS (1:10, Abnova), chicken anti-GFP (1:1,000, Abcam), mouse anti-Integrin (1:100, DSHB CE6G11), mouse anti-Dlp (1:50, DSHB 13G8), guinea pig anti-Senseless (1:1,000, gift from H. Bellen, Baylor College of Medicine, USA), mouse anti-V5 (1:500, Invitrogen), goat anti-GMAP (1:100, gift from S. Munro, MRC-LMB Cambridge, UK), guinea pig anti-Godzilla (1:1,500; ref. 22). Secondary antibodies used were Alexa 488 and Alexa 555 (1:500 for standard protocol and 1:250 for extracellular staining, Molecular Probes). Total and extracellular immunostaining of imaginal discs was performed as previously described. Imaginal discs were mounted in Vectashield with DAPI (Vector Laboratories) and imaged using either a Leica SP5 or LSM710 confocal microscope. Z stacks were generated with 1–1.5 μm intervals.

Confocal images were processed with ImageJ (NIH), Velocity (PerkinElmer), ZEN2.0 (Zeiss) and Photoshop CS5.1 (Adobe). All X–Y confocal images show a single confocal section; X–Z and Y–Z projections were created using Velocity. Adult wings were mounted in Euparal (Fisher Scientific) and imaged with a Zeiss Axioskop2 microscope with an AxioCam HRC camera.

Fluorescence in situ hybridization. L3 larvae were dissected and fixed in 4% formaldehyde-PBS, washed and quenched with 3% H2O2. Discs were then post-fixed in 3% formaldehyde–PI1ween and transferred to hybridization buffer containing the wingless probe overnight. Discs were washed and incubated with anti-DIG (Roche), and then biotinylated anti-Sheep (1:200, Jackson Laboratories), and the reaction was visualized with a Tyramide signal amplification kit (PerkinElmer). Discs were mounted in Vectashield and imaged as described above.

Drosophila boxing and clone induction. Fly stocks were kept at 18 °C, 25 °C or 29 °C on a standard medium consisting of agar, cornmeal and yeast. Crosses were performed at 25 °C unless they involved tub-Gal80°, in which case they were kept at 18 °C and moved to 29 °C when required (as indicated in the relevant figure). Gal4/UAS-RNAi and Gal4/UAS-Gal80.GFP discs were crossed at 29 °C. To inhibit endocytosis temporarily, shibire1 wandering L3 larvae were placed on a grape juice plate in a 34 °C water bath for the required time period and fixed afterwards. Endocytosis was restored by returning to 18 °C for the relevant time period before fixation. For the production of daily dlp double mutant clones (Supplementary Fig. 2), larvae were heat shocked 48 h after egg laying for 45 min at 37 °C and fixed 48 h later, at the wandering L3 stage. To generate godzilla mutant clones, MS1096-Gal4; FRT82B, godzilla1/2TM6 was crossed to UAS-GFP; FRT82B, Ubi-GFP-S65T)Jns, Rp53/TM6. To generate syb44 clones, FRT42D, Ubi-GFP/CyO was crossed to Kr/CyO; hh-Gal4, UAS-FLP/TM3ser, generating FRT42D, Ubi-GFP-S65T)Jns/CyO; hh-Gal4, UAS-FLP+/+. These were subsequently crossed to FRT42D, syb44/CyO.

Genetic modifications. To generate wg01 (F-WgGAL4, F-WgGAL4), DNA encoding OLLAS- and HA-tagged Wingless were synthesized (Genewiz) and ligated in front of 1,200 bp of 3′UTR. The tags were inserted in triplicate, separated by a Gly-Gly spacer, between Ser 306 and Gly 307. Wingless3′-3UTR and Wingless3′-3UTR were inserted sequentially in MCS and MCS3 of RIV (ref. 26; ref. 26). The resulting construct, RIV- F-WgGAL4, F-WgGAL4 was inserted into the attP site of the wg01 (ref. 6).

To generate flies allowing conditional expression of dominant-negative Godzilla, the godzilla cDNA was mutated such that two conserved histidines of the RING domain (His255 and His258) reported to be involved in the coordination of Zn2+ ions would be substituted to arginine. The resulting DNA was ligated to DNA encoding EGF to generate Godzilla.2.0 (Zeiss) and Photoshop CS5.1 (Adobe). All other Drosophila strains. shibire1 (a gift from C. O’Kane, Cambridge University, UK), FRT82B (BL 2035), FRT82B, Ubi-GFP-S65T)Jns, Rp53/TM6sb (BL 5627). FRT42D (BL 1802). FRT42D, Ubi-GFP-S65T)Jns/Cyo (BL 5628). FRT2A, Ubi-GFP (BL 1626). UAS–YFP.Rab5.588L (BL 9774). UAS–Drr2 en2.4-Gal4, UAS–EGFP (BL 2572). UAS-g2E1D2 (VDRC 19001KK), UAS-syDI4 (VDRC 10922KK), FRT2R2, godzilla144/TM3sb (ref. 22). FRT42D, syb44/Cyo (ref. 31) (a gift from C. O’Kane, Cambridge University, UK). FRT2A, dally01311, dpl01312 (ref. 32). UAS–Notum–V5 (ref. 25). UAS–Gzl.WT.GFP (ref. 22). hedgehog–Gal4 and hedgehog–Gal4, UAS–FLP/TM6 (ref. 32). hedgehog–Gal4, tub–Gal80°/TM6, generated with tub–Gal80°/TM6 (BL 7017). cg–Gal4 (BL 7011).

Drosophila genotypes listed by figures. Figure 1a–i: w1118. Figure 2a–j: wg01 (F-WgGAL4, F-WgGAL4)/UAS–Flt; hedgehog–Gal4, tub–Gal80°+. Figure 2k–r: shibire1. Figure 3a–d: UAS–GzlRNAi010100X/+; hedgehog–Gal4/UAS–mCD8.GFP. Figure 3e–f: MS1096–G4d;+; UAS–FLP/+; FRT82B, godzilla144/FRT82B, Ubi-GFP-S65T)Jns, Rp53. Figure 3g: left (control) FRT42D/FRT42D, Ubi-GFP-S65T)Jns; hedgehog–Gal4, UAS–FLP+/+. Figure 3h: FRT42D, syb44/FRT42D, Ubi-GFP-S65T)Jns; hedgehog–Gal4, UAS–FLP+/+. Figure 3i: FRT42D, syb44/FRT42D, Ubi-GFP-S65T)Jns; hedgehog–Gal4, UAS–FLP+/+. Figure 3j: FRT42D, syb44/FRT42D, Ubi-GFP-S65T)Jns; hedgehog–Gal4, UAS–FLP+/+. Figure 4a–c: UAS–Drr2–; en2.4-Gal4, UAS–EGFP/UAS–GzlRNAi010100X. Figure 4e–g: UAS–Gzl.LD.GFP+/+; hedgehog–Gal4+/+. Figure 5a: UAS–Notum–V5; CyO; tub–Gal80°+/+ at 29 °C for 15 h before fixation. Figure 5b: cg–Gal4/UAS–Notum–V5; tub–Gal80° at 29 °C for 15 h before fixation. Figure 5c: frizzled2zglRNAi–; Supplementary Fig. 4a,b: w1118. Supplementary Fig. 1: w1118. Supplementary Fig. 1b: wg01 (F-WgGAL4, F-WgGAL4)/+; hedgehog–Gal4, UAS–Flt+/+. Supplementary Fig. 1d–k: shibire1. Supplementary Fig. 2: yw hsps80+. Figure 2: FRT2A ubi-GFP/FRT2A dally01311/dpl01312. Supplementary Fig. 3a–b: UAS–GzlRNAi010100X/+; hedgehog–Gal4/UAS–mCD8.GFP. Supplementary Fig. 3c: UAS–GzlRNAi010100X/Cyo.owglacz2, hedgehog–Gal4/UAS–mCD8.GFP. Supplementary Fig. 3d: UAS–SybRNAi010100X/Cyo.owglacz2; hedgehog–Gal4/UAS–mCD8.GFP. Supplementary Fig. 4a,b: MS1096–Gal4; UAS–YFP.Rab5.588L/+; Supplementary Fig. 4c: MS1096–Gal4; UAS–Gzl.WT.GFP+/+; Supplementary Fig. 4d: UAS–Gzl.LD.GFP+/+; hedgehog–Gal4+/+. Supplementary Fig. 5: frizzled2zglRNAi–/frizzled2zglRNAi–.

31. Bhattacharya, S. et al. Members of the synaptobrevin/vesicle-associated membrane protein (VAMP) family in Drosophila are functionally interchangeable in vivo for neurotransmitter release and cell viability. Proc. Natl Acad. Sci. USA 99, 13867–13872 (2002).
32. Franck-Marro, X. et al. Glycans shunt the Wingless signal between local signaling and further transport. Development 132, 659–666 (2005).
Supplementary Figure 1 Basolateral distribution of extracellular Wingless and tools to track Wingless along the apical-basal axis. a, Cross-sectional view of a wild type imaginal disc stained with mouse anti-Wingless (red) in the absence of detergent, followed by staining with rat anti-E-Cadherin (green) in the presence of detergent. Most Wingless immunoreactivity is along the basal half of the cell surface; no signal can be detected above (apical to) adherens junctions. Scale bar represents 24 μm. b–c, Characterization of the strain used to track Wingless protein. Two cDNAs were inserted in the locus, as illustrated in Fig. 2a. In the resulting flies (wg^- insertion; F-Wg^OLLAS-F-Wg^HA)/wg^CX4), without Flp, only Wingless^OLLAS is produced and this is sufficient to sustain normal development (b). In separate experiments, we have shown that Wingless^HA is also fully functional. Expression of Flp from UAS-Flp and hedgehog-Gal4 in the posterior compartment (panel c; right hand side of disc) converts the locus to expressing Wingless^HA (green) while the control anterior compartment continues to express Wingless^OLLAS (red). This image is representative of >10 discs from 3 independent experiments. d–k, Computationally reconstructed cross sections of the imaginal discs shown in Fig. 2 k–r (hemizygous shibire6). d, Steady state distribution of extracellular Wingless along the apico-basal axis (A→B). e–g, Upon blocking endocytosis, extracellular Wingless accumulates at the apical surface and becomes depleted from the basolateral surface. h, Steady state distribution of extracellular Wingless at normal level of endocytosis activity. i, Apical accumulation and basolateral depletion upon endocytosis blockade for 60 min. j, k, The pre-blockade distribution is progressively restored upon resumption of endocytosis by transfer to 18°C. Note that panels d–k were generated from the same preparations as those shown in Fig. 2 k–r. For information on number of samples analysed, see legend of Fig. 2. Scale bars = 24 μm in a, 500 μm in b, 24 μm in c, and 16 μm in d–k.
Supplementary Figure 2 Removal of Daily and Dlp does not cause apical accumulation of extracellular Wingless in wing imaginal discs. a, daily dlp mutant clones, generated by FRT-mediated mitotic recombination and hs-Flp are depleted of Dlp protein, as expected. Mutant cells (arrows; marked by the absence of GFP) lack anti-Dlp immunoreactivity. b-e, Distribution of extracellular Wingless in and around a daily dlp double mutant clone (labeled by the absence of GFP). The same clone is shown in panels b-e with the “prime” panels showing magnification of the area flanking the Wingless strip. Lack of glypicans leads to a mild reduction of extracellular Wingless but does not cause accumulation at the apical surface (b, c), as seen in >10 discs from 3 experiments. Scale bars represent 24 μm.
**Supplementary Figure 3** Loss of Godzilla or Synaptobrevin does not alter Wingless expression in wing imaginal discs. 

**a, b.** Godzilla RNAi (hedgehog-Gal4; UAS-gzlRNAi) depletes endogenous Godzilla protein, as detected by immunofluorescence. The domain of RNAi expression is marked with GFP. Panel a shows a subapical plane and panel b an optical cross section. Scale bars represent 10 μm.  

**c.** RNAi-mediated depletion of Godzilla protein in the territory marked with GFP does not affect wingless-lacZ reporter expression (red; detected with anti-β-galactosidase).  

**d.** Depletion of syb does not alter wingless-lacZ reporter expression (hedgehog-Gal4; UAS-sybRNAi). The discs shown in c and d are representative of >10 discs from 2 experiments. Scale bars in c and d = 50 μm.
Supplementary Figure 4 Godzilla likely interacts with Wingless at rab5-positive endosomes. a, b, YFP-tagged constitutively active Rab5 (YFP.Rab5.Q88L) was expressed in the wing pouch with the MS1096-Gal4 driver. Most of the resultant enlarged apical endosomes contain Wingless and Godzilla. (b) Close-up view of Wingless-Godzilla colocalisation on the enlarged endosomes. These pictures are representative of >5 discs. Scale bars represent 20 μm (a) or 5 μm (b). c, Godzilla overexpression produces enlarged endosomes where Wingless accumulates. This disc is representative from >10 discs from 2 experiments. Scale bars represent 10 μm. d, e, Expression of GFP-tagged ligase-dead Godzilla (Gzl.LD.GFP) in the posterior compartment (hedgehog-Gal4; UAS-Gzl.LD.GFP) leads to loss of margin tissue in the posterior wing (d) Scale bar represents 500 μm. Senseless immunoreactivity is lost in the posterior compartment of 3rd instar imaginal discs of the same genotype (e). These images are representative of >10 discs from 2 independent experiments. Scale bar represents 50 μm.
**Supplementary Figure 5** Distribution of Wingless and Frizzled2. Imaginal disc from a *frizzled2<sup>GFP<sup>KI</sup></sup>* larva stained with anti-GFP (green) and anti-Wingless (red). A single focal plane (basolateral, see diagram in b') is shown. Panels b'-b''' show enlarged white box from panel a. Representative of >5 imaginal discs of the same genotype. Scale bars represent 25 μm.