

1 **Microvilli-derived Extracellular Vesicles Govern Morphogenesis in**
2 *Drosophila* wing epithelium

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14 **ABSTRACT**

15 The regulation and coordination of developmental processes involves the secretion of
16 morphogens and membrane carriers, including extracellular vesicles, which facilitate their
17 transport over long distance. The long-range activity of the Hedgehog morphogen is conveyed
18 by extracellular vesicles. However, the site and the molecular basis of their biogenesis remains
19 unknown. By combining fluorescence and electron microscopy combined with genetics and
20 cell biology approaches, we investigated the origin and the cellular mechanisms underlying
21 extracellular vesicle biogenesis, and their contribution to *Drosophila* wing disc development,
22 exploiting Hedgehog as a long-range morphogen. We show that microvilli of *Drosophila* wing
23 disc epithelium are the site of generation of small extracellular vesicles that transport Hedgehog
24 across the tissue. This process requires the Prominin-like protein, whose activity, together with

25 interacting cytoskeleton components and lipids, is critical for maintaining microvilli integrity
26 and function in secretion. Our results provide the first evidence that microvilli-derived
27 extracellular vesicles contribute to Hedgehog long-range signaling activity highlighting their
28 physiological significance in tissue development *in vivo*.

29

30 Hedgehog (Hh) is a well-known long-range morphogen controlling tissue patterning
31 and cell differentiation during embryonic development¹. In the *Drosophila* wing imaginal disc
32 epithelium, Hedgehog produced in posterior cells, signals across the anterior (A)/posterior (P)
33 boundary to induce the expression of short- [i.e. *engrailed* (En), *Patched* (ptc)], and long-range
34 [i.e. *decapentaplegic* (*dpp*)] target genes in anterior recipient cells. Different modes of carrier-
35 mediated Hh long-range signaling have been proposed¹, including extracellular vesicles (EVs),
36 that arise either from fusion of multivesicular body (MVB) with the plasma membrane
37 (exosomes), or from budding of the plasma membrane (ectosomes also termed microvesicles².
38 Evidences have been provided to suggest that both types of EVs, whose nature is tightly
39 dependent on the site of Hh secretion (apical³⁻⁵ and/or basolateral secretion⁶) could mediate Hh
40 long-range activity in *Drosophila* epithelia^{4,7-9}. The apical membrane of epithelial cells is
41 characterized by the presence of microvilli that have been shown to give rise to EVs^{10,11}.
42 However, our understanding of the mechanisms of microvilli-EV biogenesis, and their potential
43 contribution to intercellular communication during development is presently unclear. This is
44 mainly due to the fact that current models describing the origin of microvilli-derived EVs, focus
45 on the relationship between the occurrence of microvilli-derived EVs and specific
46 developmental stages¹⁰. These studies, which are based on ultrastructural, biochemical and
47 pharmacological observations, reveal only part of the mechanisms of EV biogenesis and do not
48 directly investigate the functional consequences during the developmental process *in vivo*. To
49 understand the relationship between microvilli, EV biogenesis and their potential significance

50 in development, we investigated the origin of EVs, the molecular mechanisms underlying their
51 biogenesis, and their function in Hh long-range signaling using *Drosophila* wing imaginal disc
52 epithelium as a paradigm.

53

54 Microvilli that are globally aligned at the apical membrane of epithelial cells, bear
55 proteins of the Prominin family¹²⁻¹⁴, and have been shown, in mammalian cells, to give rise to
56 Prominin-containing EVs^{10,11}. Considering this, we examined whether the sorting of Hh to
57 microvilli and their ability to generate EVs could be prerequisites for the EV-mediated
58 deployment of Hh long-range signaling in the *Drosophila* wing imaginal disc (Fig. 1a). To
59 investigate the formation of microvilli-derived EVs, we monitored the Prominin-like (PromL)
60 protein that has been reported, upon overexpression, to distribute to apical protrusions of
61 *Drosophila* wing imaginal discs¹⁵. To validate the newly generated antibodies raised against
62 the PromL protein (methods and Supplementary Fig. 1a), we analyzed their subcellular
63 distribution in the dorsal wing disc compartment expressing GFP-tagged PromL¹⁵ driven by
64 *apterous-Gal4 driver* (*ap>PromL-GFP*) and compared to the ventral wild-type compartment
65 within the same discs. Endogenous PromL signals massively colocalized with PromL-GFP,
66 distributed at the uppermost apical surface above aPKC, E-cadherin (E-cad) and Dlg (apical,
67 subapical and basolateral markers respectively), was not detected at the basolateral where
68 PromL-GFP was previously observed¹⁵, and did not overlap with GFP-tagged Viking (GFP-
69 Vkg, a collagen IV type molecule that labels the basement membrane)¹⁶ (Fig. 1b;
70 Supplementary Fig 1b,c). In addition, PromL fully colocalized with Cad99c, a specific marker
71 of microvilli of ovarian follicle and wing imaginal disc epithelial cells^{17,18} (Supplementary Fig.
72 1d). Overall, these results show that PromL, is associated with the apical compartment of
73 epithelial wing disc cells, consistent with an association and distribution to microvilli.

74 Next, we functionally tested the impact of PromL protein on microvilli architecture and
75 integrity through knock down (KD) using RNA interference (RNAi) driven by *ap>* or
76 *hedgehog-Gal4 (hh>)* to target dorsal or posterior cells respectively. We found that in both
77 genetic backgrounds PromL signal was specifically abrogated (Fig. 1b; Supplementary Fig. 2a).
78 However, cell polarity (as revealed by aPKC, E-cad and Dlg staining) was not perturbed and
79 apoptosis levels were not increased (as revealed by caspase III staining), indicating an absence
80 of tissue stress in such condition (Fig. 1b; Supplementary Fig. 2b,c). In addition, the *wt*
81 compartment, displayed the usual dot-like pattern of PromL reflecting a correct organization of
82 microvilli at the apical plasma membrane (Fig. 1a,b; Supplementary Fig. 1e). We conclude that
83 PromL specifically localised and confined to microvilli in the wing disc epithelium.

84 Upon a closer inspection of the wing imaginal disc epithelium at the ultrastructural level
85 using transmission electron microscopy (TEM), we noticed that posterior cells of *hh>PromL*
86 *RNAi* wing discs displayed abnormal microvilli or severely atrophic protrusions (with a length
87 98% shorter than that of microvilli from wild-type (*wt*) discs) (Fig. 1c,d; Supplementary Fig.
88 3a-c). In contrast, in anterior *wt* cells, microvilli length, density and morphology was similar to
89 that of *wt* discs (Fig. 1c,d; Supplementary Fig. 4 a,b). These findings point to the requirement
90 of PromL for the maintenance of the proper microvilli architecture and integrity in the wing
91 disc epithelium, and are consistent with recent observation that the homologous Prominin-1
92 modulates microvilli architecture in mammalian cells¹⁹. Furthermore, our results establish that
93 members of the Prominin protein family are key determinants of microvilli formation and
94 integrity, putting forward the existence of a conserved function of the Prominin protein family
95 through evolution.

96 Having established that PromL localises to and is required for the maintenance and
97 integrity of microvilli, we explored its relationship with Hh localization and its signaling
98 properties. We first examined Hh distribution in *wt* and in posterior (*hh>*) or dorsal (*ap>*)

99 *PromL* depleted compartments of wing imaginal discs, and found that in *wt* discs, Hh
100 distributed at the uppermost apical surface above E-cad (Fig. 1e). No such distribution was
101 observed in *PromL* mutant compartment in which microvilli were absent or misshapen (Fig.
102 1c), and lacking therefore a structural support for the apical distribution of Hh. Consistently, in
103 *PromL* depleted cells, Hh was detected at the basolateral at the level of Dlg, and subapical to
104 E-cad (Fig. 1f; Supplementary Fig. 2b). A subsequent quantitative analysis of the signal
105 distribution revealed a substantial reduction of Hh staining at microvilli in posterior cells (Fig.
106 1g,h; methods), further supporting that the apical distribution of Hh relies on the presence of
107 intact microvilli.

108 To examine the consequences of the subcellular redistribution of Hh in *PromL* depleted
109 cells interferes on Hh signaling properties, we next monitored the expression of long- and short-
110 range Hh target genes simultaneously in *hh>PromL RNAi* discs. In wing imaginal discs, *dpp-*
111 *lacZ* reporter expression reflects the apical secretion and the long-range Hh signaling, whereas
112 En and Ptc expression is attributed to Hh short-range activity mediated by its basolateral
113 secretion³⁻⁵. We found that in *PromL* KD discs Hh long-range signaling is impaired, as
114 evidenced by a severe decrease of *dpp-lacZ* reporter expression ranging from 60-65%. In
115 contrast the expression of the short-range target genes, En and Ptc, remained unaltered (Fig. 1i,
116 j; Table 1; Supplementary Fig. 2d). These results consistently establish that *PromL* and hence
117 intact microvilli, are not only required for the apical distribution of Hh but also critically
118 involved in Hh long-range signaling in the wing disc epithelium.

119 To further validate this hypothesis, and substantiate the requirement of microvilli
120 integrity for Hh signaling as suggested by our observations (Fig. 1b-j), we chose to analyze the
121 effect of Dispatched (Disp), a positive regulator of Hh secretion and trafficking^{5,6,20}, on the
122 microvillar localisation of Hh. We reasoned that in *disp* mutant discs, in which Hh is retained
123 in producing cells, and *dpp-lacZ* expression is severely restricted (Fig. 2a; Table 1), the

124 distribution of Hh to microvilli might also be impaired. An analysis of Hh signal showed that
125 in *disp* mutant discs Hh consistently distributed to the apical, at the level of E-cad, but not Hh
126 to microvilli (Fig. 2b,d), at odds with *wt* discs, in which Hh distributed to microvilli and
127 colocalised with PromL (50% of the uppermost apical Hh signal) (Fig. 2c,d). Accordingly,
128 long-range Hh signaling is impaired in *disp* mutants (Fig. 2a). We also noticed that PromL
129 signal, in anterior and posterior compartment, was more punctate and irregularly spiky, pointing
130 to a shortening of microvilli (Fig. 2b). Additional ultrastructural analysis of *disp* mutant discs
131 indeed showed shorter and far less microvilli (66% of that of *wt*), with an average of 2.9 ± 1.16
132 microvilli per cell (Fig. 2d; Supplementary Fig. 3c). Together these experiments indicate that
133 in addition to its role on Hh secretion and trafficking, Disp modulates microvilli architecture,
134 reinforcing again the idea that a proper organization of apical membrane and the maintenance
135 of microvilli architecture is critical for Hh-long-range signaling.

136 The maintenance of the apical plasma membrane structure relies on the asymmetrical
137 distribution of specific phospholipids between the outer and inner leaflets of plasma
138 membranes²¹. This is controlled by ATP8B, a putative aminophospholipid translocase, whose
139 depletion perturbs apical membrane organization, stereocilia and microvilli integrity^{22,23}. It is
140 thus expected that abrogation of ATP8 activity can perturb microvilli organization and hinder
141 Hh long-range signaling also in the wing imaginal disc. Consistent with this hypothesis, the
142 qualitative and quantitative TEM analysis revealed that ATP8B depletion in posterior cells
143 (*hh>ATPB8 RNAi*), resulted in an alteration of microvilli number, length, and morphology, and
144 perturbed the characteristic dot-like staining pattern of PromL indicative of the proper
145 microvilli integrity (Fig. 3a-c; Supplementary Fig. 1e). This was accompanied by a subapical
146 redistribution of Hh, an impediment of PromL/Hh colocalisation, and a reduction of *dpp*
147 expression in recipient cells as a consequence of impaired long-range Hh signaling, but no effect
148 of En and Ptc expression was observed (Fig. 3c-e; Table 1). However, ATP8B depleted cells

149 show well developed adherent junctions, confirming their ability to polarize (Fig. 3a). Hence,
150 loss of ATP8B activity may affect the proper distribution of PromL to microvilli, by altering
151 the phospholipid composition of the plasma membrane, or by potentially decreasing the levels
152 of the plasma membrane phospholipid phosphatidylinositol 4,5-bisphosphate (PIP₂)²⁴.

153 As PIP₂ is enriched at the apical plasma membrane of epithelial cells, regulates the
154 interaction of signaling proteins with actin-binding proteins, and promotes the bundling
155 function of villin, it could thereby modulate the formation of actin filaments structures and
156 cellular protrusions, including microvilli (Kumar). To test this possibility, we silenced two
157 actin-binding proteins, Quail (Qua) (*ap>Qua RNAi*), the *Drosophila* villin-like protein²⁵, or
158 Fimbrin (Fim) (*hh>Fim RNAi*) in *Drosophila* imaginal discs. In both conditions, PromL levels
159 were drastically reduced (Fig. 3 f,g), and microvilli, when present, were strongly defective (Fig.
160 3b,d,h,i; Supplementary Fig. 4c). As expected, Hh long-range signaling was impaired to similar
161 extents in both genetic backgrounds, as confirmed by the subapical distribution of Hh and by
162 the reduced *dpp* expression in target cells (Fig 3f,g,j; Supplementary Fig. 3; Table 1). This
163 indicates that the disassembly of actin filaments caused profound structural alterations on the
164 microvillar architecture and put forward the positive contribution of actin cross-linking factors
165 in this process. Moreover, this reveals that an active interplay between PromL and cytoskeleton
166 components contributes to the biogenesis and maintenance of microvilli that are critical for Hh-
167 long-range signaling.

168 How do microvilli contribute to Hh long-range signaling? While previous work has
169 established that microvilli give rise to Prominin-containing EVs^{10,11}, their contribution to
170 intercellular communication during development was never directly demonstrated. Considering
171 the requirement of microvilli for Hh long-range signaling (as shown above), we reasoned that
172 EVs could bud from microvilli and serve as a means to transport Hh to distant recipient cells.
173 To test this assumption, we performed an EM analysis of serial sectioning of *wt* wing imaginal

174 discs epithelial cells. We noticed the existence of small vesicular structures of 60 to 150 nm
175 diameter, often clustered at the vicinity of microvilli, but also free within the lumen (Figure 4
176 a,b; Supplementary 4d; methods). We also observed that MVB with intraluminal vesicles (ILV)
177 of smaller diameter (25-40 nm), were consistently found at close vicinity of the basolateral
178 plasma membranes (Fig. 4a, lower right panel; Supplementary 4e), but not at the apical site,
179 indicating that MVBs may not be prone to an apical secretion of “endosome-derived”
180 exosomes². Importantly, such vesicular structures were not detected in discs depleted for
181 PromL, ATP8B, Qua, Fim, or *disp* mutant discs, in which microvilli are absent or atrophic, and
182 *dpp-lacZ* expression is substantially decreased (Fig. 1c, 2e, 3a,h,i; Table 1), underscoring the
183 correlation between the presence of microvilli, the occurrence of these secreted vesicles and Hh
184 long-range activity. A closer inspection of the microvilli in *wt* discs, revealed the presence of
185 buds at the microvillar membrane (Fig. 4c). Electron tomography and 3D-reconstructions of
186 the apical plasma membrane confirmed the existence of buds still connected to the microvillar
187 membrane, but also isolated free vesicles within the extracellular space certainly after fission
188 (Fig. 4d, Supplementary Movies 1 and 2). Together, these results substantiate microvilli as a
189 site of EV biogenesis and demonstrate that the active interplay between PromL, actin cross-
190 linking proteins, and microvilli lipids is critical for the maintenance of microvilli integrity and
191 the biogenesis of EVs. Consistent with these findings, Prominin-1 containing EVs were shown
192 to originate from the microvilli membrane and cilia of neuroepithelial and neural progenitors
193 cells respectively^{10,26}. In agreement with our findings, actin mediate EV release from the tip of
194 cilia²⁷, and dATP8B protein is concentrated in the cilia of olfactory neuron dendrites²⁸ where
195 it could play similar roles as the one shown here in microvilli.

196 Given that PromL is critical for microvilli biogenesis, and that Hh distributed to
197 microvilli where it colocalises with PromL (Fig. 1g,h; 2c,d; 3d), we reasoned that an increase
198 of Hh long-range activity might be correlated with an increase of EV biogenesis. To test this

199 assumption, we examined the consequences of the overexpression of PromL-GFP in Hh
200 producing cells (*hh>PromL-GFP*). EM analysis revealed a substantially increased of EV
201 number (35% as compare to *wt*) in PromL-GFP discs, which resulted in a slight but not
202 significant reduction in microvilli number and length (Fig. 4e,f; Supplementary Fig. 4b). We
203 also found that overexpression of PromL-GFP promoted a massive Hh release into the lumen,
204 which correlated with a significant expansion of the *dpp* expression domain (Fig. 4g,h; Table
205 1). These results are consistent with our hypothesis and emphasize the interdependence between
206 PromL localisation to microvilli, EV biogenesis and Hh long-range activity.

207 In light of these results, we anticipate that microvilli-derived EVs, containing both Hh
208 and PromL protein are released into the extracellular medium. To validate this hypothesis, we
209 investigated their occurrence and their potential properties in wing discs overexpressing
210 PromL-GFP and Hh tagged with red fluorescent protein (Hh-RFP) in Hh producing cells
211 (*hh>Hh RFP; PromL GFP*). In a first round of experiments, we set out to investigate the
212 distribution of Hh-GFP and Prom-GFP using immunoelectron microscopy (IEM), for a
213 qualitative analysis. In agreement with our above observations (Fig. 1g,h; 2c,d; 3d,) both
214 proteins localised not only to microvilli (Fig. 5a), but also on EVs (Fig. 5 b). To elucidate their
215 dynamics and their functional roles, we investigated their properties *in vivo*. Live imaging of
216 *hh>Hh RFP; PromL GFP* discs revealed that the majority (80%) of EVs released by the
217 posterior compartment was positive for Hh-RFP and Prom-GFP, whereas the others only
218 displayed Hh-RFP (Fig. 5c-e). These EVs were not static but engaged in a dynamic movement
219 and trafficked associated to one another within the extracellular space (Fig. 5f,g; supplementary
220 Fig. 5a; Supplementary Movies 3). These EVs are very likely the extracellular carriers of Hh,
221 facilitating thereby its long-range activity. These results are consistent with our hypothesis that
222 microvilli of the wing disc epithelium are the site of generation of small EVs, that are critically
223 involved in the transport of Hedgehog across to facilitate its long-range activity.

224 Understanding the origin and function of EVs *in vivo* during development has so far
225 been limited especially because of a shortage of methods suitable to demonstrate causality *in*
226 *vivo*^{10,11}. Taking advantage of complementary electron and fluorescence microscopy, live
227 imaging, cell biology and genetic approaches, our experiments unveil that EVs provide a means
228 for exchanging signaling cues between cells at distance. Here, we find that PromL, by
229 interaction with lipids and cytoskeleton components, is a key determinant of microvilli
230 formation *in vivo*, whose integrity is critical for the biogenesis of EVs and their signaling role
231 (Fig. 1; Fig. 3). Finally, we uncover that microvilli are the preferential site for the generation
232 of small EVs conveying Hh, - different from exosomes- , revealing the existence of a new
233 potential mechanism mediating Hh long-range signaling in the *Drosophila* wing imaginal disc
234 epithelium, and unveil their physiological significance in tissue development *in vivo*.

235 Considering that the morphogen Hh controls a variety of conserved functions in
236 invertebrates and vertebrates, and that the dysregulation of the Hh pathway promotes
237 developmental defects and contributes to several cancer types, it will be of high interest to
238 investigate the contribution of the EV-mediated Hh signaling described in this study to such
239 physiological and pathological processes.

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336 **Authors contributions**

337 Conceptualization: P.P.T, G.R, and G.D.; Methodology: I.H, M.R, L.S., L.R., and G.D.;
338 Software: A.S.M.; Formal analysis: I.H, A.S.M, and G.D.; Investigation: I.H., M.R., L.R., G.D.;
339 Resources: R.B., P.P.T., G.R.; Writing - Original Draft: G.D.; Writing - Review & Editing:
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341

342 **Competing interests**

343 The authors declare no competing interests.

344

345 **Data and materials availability**

346 all data is available in the manuscript or the supplementary information.

347

348 **Figure legends**

349 **Figure 1. Hh long-range activity relies on microvilli integrity.** **a**, Schematic of the larval
350 wing imaginal disc epithelium and a transverse section. The disc is oriented anterior (A) to the
351 left, posterior (P) to the right. The wing imaginal disc consists of two layers of cells with apical
352 surfaces facing each other: the columnar epithelium (CE) of the wing disc pouch overlaid
353 by the squamous epithelium (SE), separated by the extracellular space (lumen). Microvilli cover
354 the apical surface of the wing imaginal disc. **b, e-g, i,j** Single confocal transverse and XY
355 sections of discs of the indicated genotypes stained for PromL (red; white), aPKC (green), E-
356 cad (blue), Hh (green), En (blue; white), Ptc (red; white) and β -gal (reflecting the expression
357 of the reporter gene *dpp-lacZ*; green; white). **b**, Yellow arrows mark the absence of PromL in
358 D compartment. Yellow broken line delineates the apical surface. **c**, Transmission electron
359 micrograph of *hh>PromL RNAi* wing disc showing the absence of microvilli PromL depleted
360 P compartment (arrows). Asterisks point to adherent junctions. **d**, Graph of microvilli length
361 for the indicated genotypes (see Supplementary Fig. 4a). Mean \pm SEM analysed by unpaired *t*
362 test, **** $p < 0.0001$. **e, f**, Yellow arrows and arrowhead mark apical and subapical Hh
363 distribution in *wt* and *hh>PromL RNAi* wing discs respectively. **g**, Distribution of Hh, and
364 PromL in *wt* discs. **h**, Quantification of PromL and Hh colocalisation within the delimited
365 region (see Supplementary information). Mean \pm SEM analysed by unpaired *t* test (**** $p <$
366 0.0001) ($n=24$ uppermost apical regions, 8 discs). **i,j**, Confocal XY single section showing Hh
367 target gene expression. Red dots in the insets depict the number of cells expressing En, Ptc and
368 *dpp-lacZ* for the shown discs, but representative of all examined discs (*wt* $n = 8$; PromL RNAi
369 $n = 6$) (see Table 1). Broken lines delimit V/D and A/P compartments. Scale bars: $1\mu\text{m}$ (**c**); 20
370 μm (**b, e-h**).

371

372 **Figure 2. In *disp* mutant discs microvilli integrity is perturbed.** **a-c**, Single confocal
373 transverse and XY sections of discs of the indicated genotypes stained for Hh (green; white),

374 β -gal (red; white), PromL (red; white), and E-cad (blue). **a**, Hh is retained in P compartment
375 and *dpp-lacZ* expression is reduced to 4 cells (red dots in the inset and Table 1). **b**, Hh distributes
376 at the apical at the level of E-cad (white arrow). Yellow arrows point to PromL staining
377 reflecting changes in microvillus length, and compare to PromL staining in A *wt* compartment
378 typical of the intact microvilli organization (**c**). Yellow arrows point to the similar PromL signal
379 in A and P compartments. **c**, In *wt* discs, Hh distributed to microvilli. Bottom: shows the
380 colocalisation of both proteins (see Supplementary information). **d**, Quantification of
381 PromL/Hh colocalisation (see Supplementary information). Mean \pm SEM analysed by unpaired
382 *t* test (**** $p < 0.0001$) ($n = 18$; 6 discs). **e**, Transmission electron micrograph showing shorter
383 microvilli (black arrows) as compare to *wt* (Fig. 4a). Asterisks depict adherent junctions. **f**,
384 Graph reporting microvilli length. Mean \pm SEM analysed by unpaired *t* test (**** $p < .0.0001$).
385 Scale bar: 20 μ m; 1 μ m (**e**). White dashed line delimits A/P compartments.

386

387 **Figure 3. Lipids and cytoskeleton components contribute to microvilli architecture. a,h,i**,
388 Transmission electron micrograph of transverse sections of wing imaginal discs of the indicated
389 genotype showing microvilli in A (black arrows), and adherent junctions (asterisk). Note that
390 in P compartment, microvilli are reduced in size, or absent. **b**, Histogram of microvilli length.
391 Mean \pm SEM analysed by unpaired *t* test (**** $p < .0.0001$). n.s., not significant, $p = 0.5230$.
392 **c,e-g,j**, Transverse (**c,f,g**) and XY (**e,j**) confocal sections of wing imaginal discs from the
393 indicated genotypes stained for PromL (red; white), Hh (green; white), En (blue, white), Ptc
394 (red; white), β -gal (green, white), *wg* (blue). **c,f,g**, Distribution of Hh and PromL for the
395 indicated genotypes. **c**, Note absence of colocalisation of PromL and Hh in ATP8B depleted
396 cells (white arrow). **f,g**, White and yellow arrows depict the absence of PromL staining and the
397 basolateral localisation of Hh in D PromL depleted cells respectively. **d**, Quantification of
398 PromL/Hh colocalisation in discs of the indicated genotypes. Mean \pm SEM analysed by

399 unpaired *t* test (**** $p < 0.0001$). Between 8-15 discs were analysed. **e,j**, Hh target gene
400 expression in discs of the indicated genotypes. Red dots in the insets indicate the cells
401 expressing En, Ptc and *dpp-lacZ* for the shown discs but representative of all examined discs
402 (*ATP8B RNAi* $n = 5$; *Quail RNAi* $n = 7$; *Fim RNAi* $n = 6$) (see Table 1). Black (**a,h,i**) and white
403 broken lines (**c,e-g,j**) delimit the A/P or V/D compartments. Scale bar: 1 μm (A, B); 10 μm (C-
404 L); 20 μm (M).

405

406 **Figure 4. Microvilli-derived EVs control Hh long-range signaling.** (**a,c,e**) Transmission
407 electron micrograph images of a transverse section of wing imaginal discs. **a**, Microvilli (black
408 arrows), adherent junction (red arrowhead). Top right: magnification of the white boxed region
409 showing vesicular structures at the vicinity of the microvilli (asterisks). Bottom right shows an
410 MVB. Yellow arrows point to ILVs. **b**, Distribution of EVs based on their diameter size ($n =$
411 32). **c**, EVs are close to microvilli (asterisks), where a bud is also visible (yellow arrow). Note
412 that EVs are less electron dense than sections of microvilli (black arrowheads). **d**, 3D-
413 reconstruction and modelisation of vesicles budding at microvilli. The buds (red) are still
414 connected to the microvillar membrane (yellow) and free vesicles (red) are detected into the
415 lumen. Right, vesicles 1-3 budding at microvilli correspond to the vesicles in (**d**). **e**,
416 Transmission electron micrograph of *hh>PromL-GFP* disc showing EVs (yellow asterisks) into
417 the lumen and close to microvilli (yellow asterisks). **f**, Histogram reporting the length of
418 microvilli. Mean \pm SEM analysed by unpaired *t* test (n.s., not significant), $p = 0.4474$. (**g,h**)
419 *ap>PromL-GFP* (**g**) or *hh> PromL-GFP* (**h**) discs stained for E-cad (blue), Hh (red), GFP
420 (green), and β -gal (red, white). (**g**) Hh (yellow arrow) is detected within the lumen (delimited
421 by a white dotted line) in both D and V compartments. (**h**) XY confocal section. Left, PromL-
422 GFP is detected in A cells (yellow arrow). Right, cells expressing *dpp-lacZ* (red dots) in the

423 inset. Six to eight discs from each genotype were analysed, (see Table 1). Broken lines delimit
 424 the V/D (**g**) or A/P (**h**) compartments. Scale bar: 0.2 nm (**a,c-e**); 20 μ m (**g,h**).

425

426 **Figure 5. Hh-PromL-EVs move into the extracellular field.** **a**, Ultrathin cryosection of
 427 *hh>Hh RFP; PromL-GFP* disc immunolabeled for Hh (PAG 10; black arrows) and GFP (PAG
 428 15, yellow arrows) showing both proteins at microvilli. **b**, EVs detected into the lumen are
 429 positive for Hh (black arrow), and PromL (yellow arrow). **c**, Maximum intensity z-projection
 430 of 167 slices spaced 1 μ m apart of *hh> Hh-RFP; PromL-GFP* disc recorded every 3 min.
 431 Plasma membrane was stained with Cellmask (Cyan). **d**, Magnified area of z-projections of 167
 432 plans, spaced 1 μ m apart, displaying a XY view of the anterior compartment of *hh> Hh-RFP;*
 433 *PromL-GFP* disc recorded every 3 min for 90 min. Bottom, magnification of the inset showing
 434 examples of Hh/PromL-containing EVs (white circles) or Hh-containing EVs (yellow circles).
 435 **e**, Histogram of Hh/PromL-EVs or Hh-EVs counted in the anterior compartment at different
 436 times and in different regions, (n = 894). **f**, Frames from Movie 2 of *hh>Hh-RFP; PromL-GFP*
 437 disc at the indicated time-points. **g**, The travel distance of Hh/PromL-EVs (white arrowheads)
 438 gradually increased with time and is of 10 μ m in 80 min for the shown vesicle. The manual
 439 tracking of EVs was performed in the boxed region in (**c**). Scale bar: 0.2 μ m (**a,b**); 20 μ m (**c,f**);
 440 5 μ m (**d,f**).

441

442 **Table 1. En, Ptc and ddp-lacZ expression in different genotypes.**

443

444

Genotype	Mean number of cells expressing genes		
	En	Ptc	dpp-lacZ
<i>wt</i> (n=12)	2(\pm 0.42)	3.7(\pm 0.4)	7.6 (\pm 0.5)
<i>hh>PromL RNAi</i> ^{51957GD}	2 ^b	3.8 ^b (\pm 0.3)	2.6 ^a (\pm 0.5)
<i>hh>PromL RNAi</i> ^{102612KK}	2 ^b (\pm 0.4)	3.7 ^b (\pm 0.4)	3 ^a (\pm 0.3)
<i>disp</i> ^{SO37707}	n.d.	n.d.	2.5 ^a (\pm 0.5)
<i>hh>ATP8B RNAi</i> ^{102648KK}	1.92 ^b (\pm 0.29)	3.7 ^b (\pm 0.4)	4 ^a (\pm 0.3)

450

451	hh>Qua RNAiGD ^{51957GD}	1.92 ^b (± 0.3)	4 ^b	3.8 ^a (± 0.3)
452	hh>Qua RNAi ^{100856KK}	1.91 ^b (± 0.3)	3.8 ^b (± 0.3)	4 ^a (± 0.3)
453	hh>Fim RNAi ^{47514GD}	1.9 ^b (± 0.3)	3.9 ^b (± 0.4)	4 ^a (± 0.3)
454	hh>Fim RNAi ^{47511GD}	1.97 ^b (± 0.3)	3.8(± 0.4)	4.2 ^a (± 0.4)
455	hh>Fim RNAi ^{46028GD}	2.2 ^b (± 0.5)	3.9 ^b (± 0.3)	4.1 ^a (± 0.4)
456	hh>Fim RNAi ^{46029GD}	1.97 ^b (± 0.3)	3.7 ^b (± 0.4)	4.1 ^a (± 0.4)
457	hh>UASPromL GFP	n.d.	3.9 ^b (± 0.2)	10.3 ^a (± 0.4)
458				

459 The number of cells expressing En, Ptc and dpp-lacZ was assessed in the rectangles in Fig. 1i,j;
460 Fig. 2a; Fig. 3e,j; Fig.4h, Supplementary Fig. 2d; Supplementary Fig. 3a-e) in at least 8 different
461 discs for each genotypes. Mean ± SEM analyzed by unpaired *t* test: ^a (**** *p* < 0.0001); ^b (n.s.,
462 not significant).n.d. not determined.

Figure 1

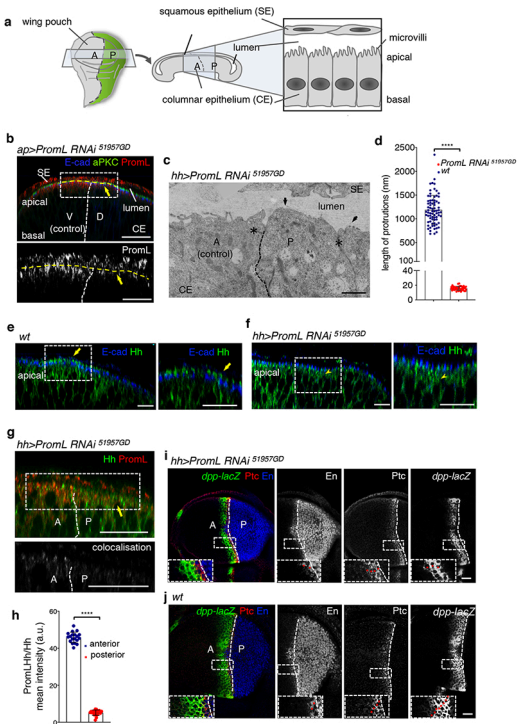


Figure 2

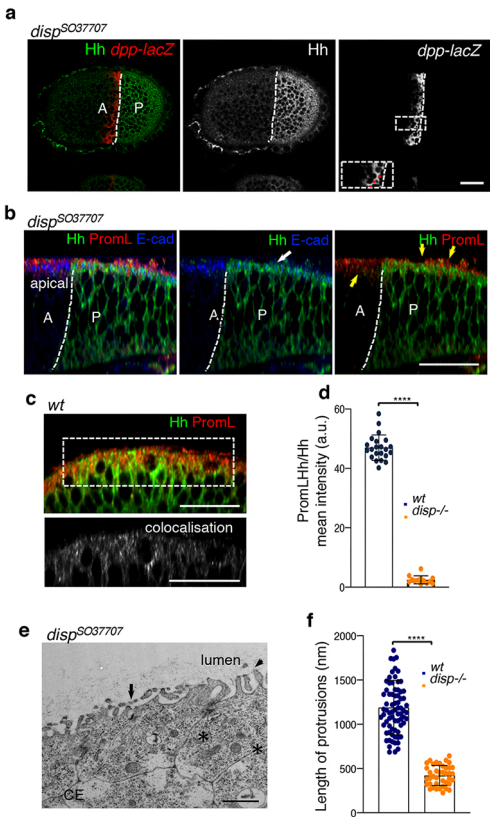


Figure 3

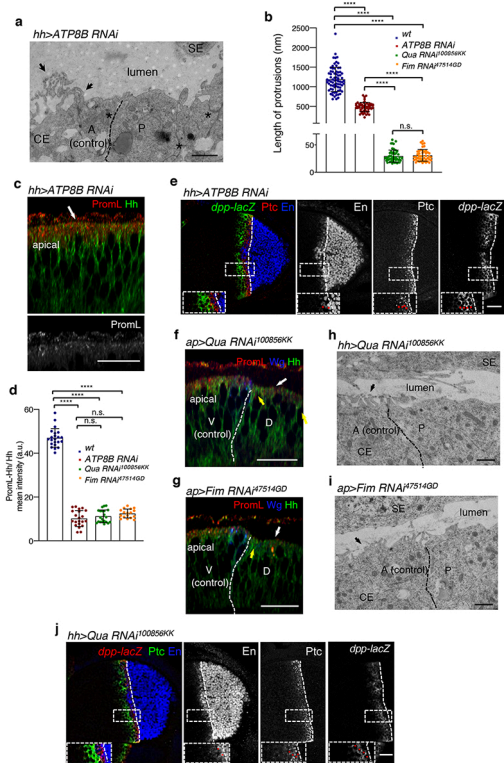


Figure 4

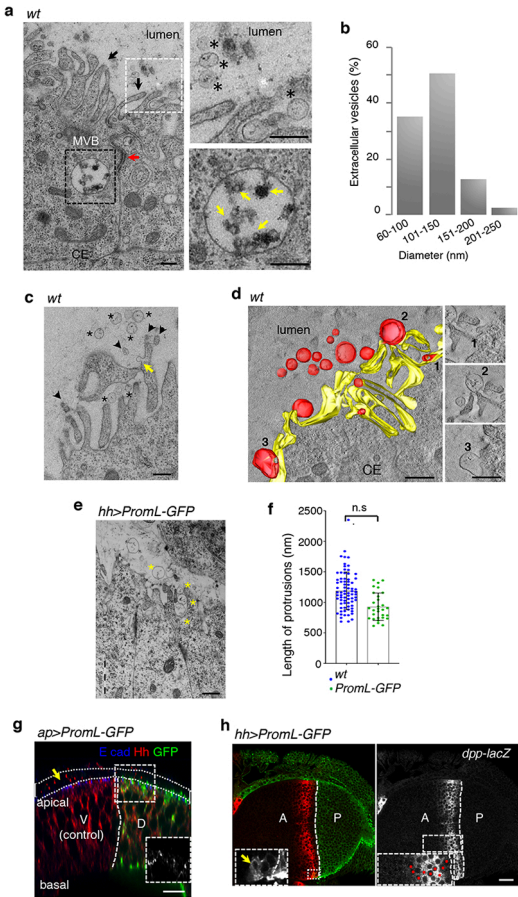
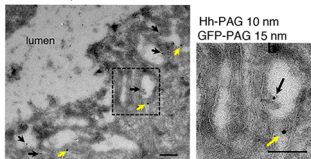
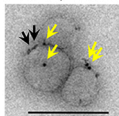


Figure 5

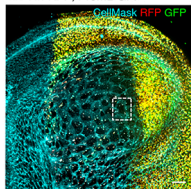
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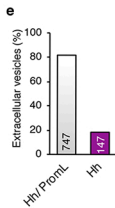
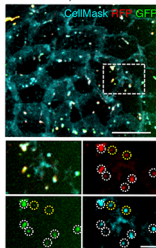
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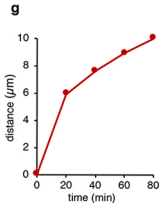
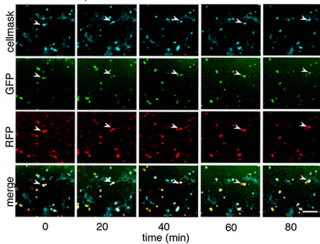
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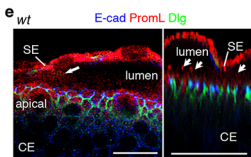
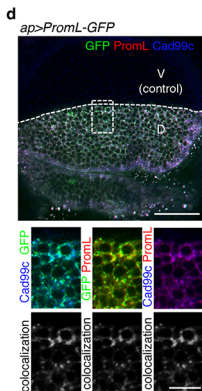
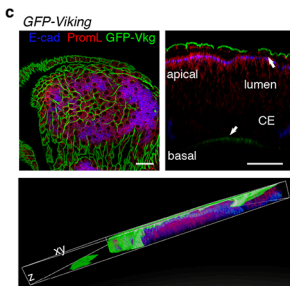
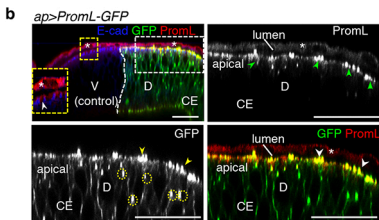
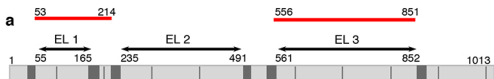
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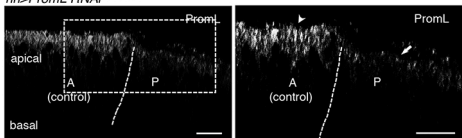
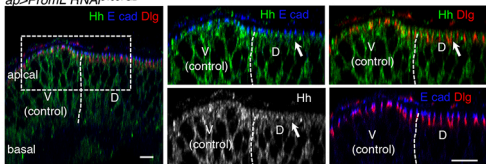
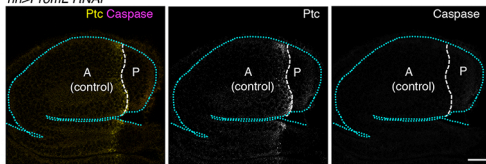
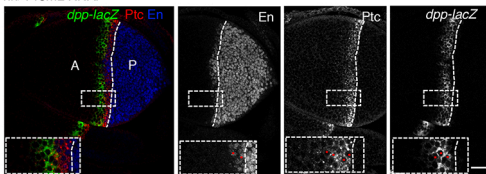


f *hh>Hh-RFP; PromL-GFP*

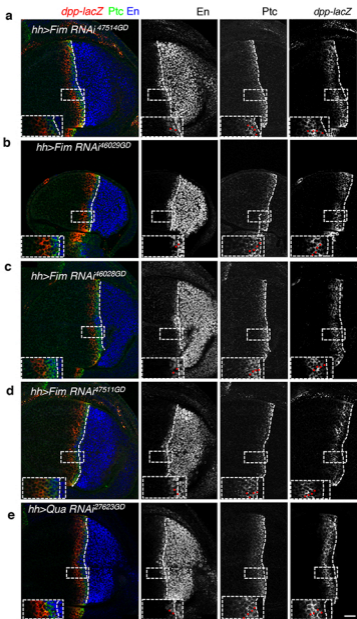


Supplementary Figure 1

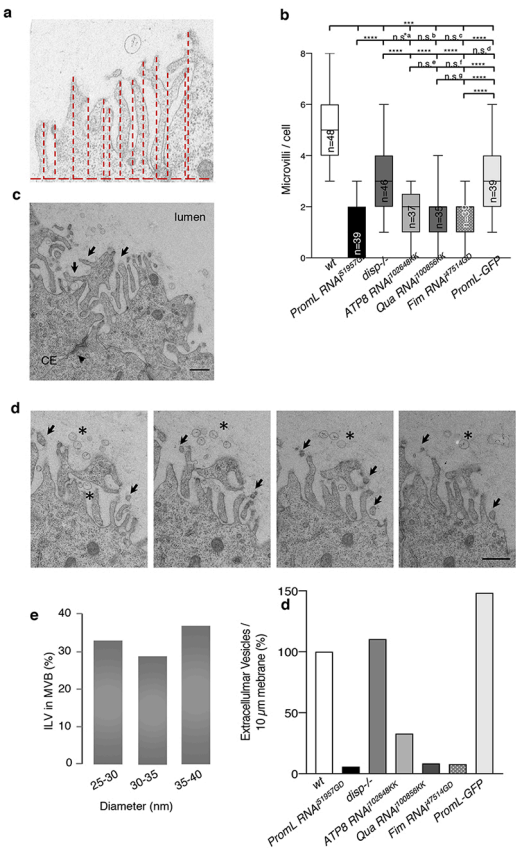


a*hh>PromL RNAi^{51957GD}***b***ap>PromL RNAi^{51957GD}***c***hh>PromL RNAi^{51957GD}***d***hh>PromL RNAi^{102648KK}*

Supplementary Figure 3



Supplementary Figure 4



Supplementary Figure 5

