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ARTICLE

Wg secreted by conventional Golgi transport diffuses and forms Wg gradient whereas Wg tethered to extracellular vesicles do not diffuse

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Abstracts

Wingless (Wg)/Wnt family proteins are essential for animal development and adult homeostasis. *Drosophila* Wg secreted from the dorsal-ventral (DV) midline in wing discs forms a concentration gradient that is shaped by diffusion rate and stability of Wg. To understand how the gradient of extracellular Wg is generated, we compared the secretion route of NRT-Wg, an artificial membrane-tethered form of Wg that is supposedly not secreted but still supports fly development, to that of wild-type Wg. We found that wild-type Wg is secreted by both conventional Golgi transport and via extracellular vesicles (EVs), and NRT-Wg can be also secreted via EVs. Furthermore, wild-type Wg secreted by Golgi transport diffused and formed Wg gradient but Wg-containing EVs did not diffuse at all. In case of Wg stability, Sol narae (Sona), a metalloprotease that cleaves Wg, contributes to generate a steep Wg gradient. Interestingly, Wg was also produced in the presumptive wing blade region, which indicates that NRT-Wg on EVs expressed in the blade allows the blade cells to proliferate and differentiate without Wg diffused from the DV midline. We propose that EV-associated Wg induces Wg signaling in autocrine and juxtaposed manners whereas Wg secreted by Golgi transport forms gradient and acts in the long-range signaling, and different organs differentially utilize these two types of Wg signaling for their own development.

Introduction

Wnt family is a group of essential morphogens for animal development and homeostasis by governing multiple cellular functions such as cell proliferation, differentiation, cell survival, stem cell maintenance, and cell polarity [1–5]. Secreted Wnts form a concentration gradient that is shaped by diffusion and stability of Wnt [6]. This Wnt gradient is essential for embryonic muscle development, tail formation, and tissue polarity because the transcriptional induction of Wnt-responding genes is dependent on the concentration of gradient along the axis [7–10]. Fly Wg, a homolog of mammalian Wnt-1 [11–14], and the fly wing disc have been used to study Wnt gradient because Wg is heavily expressed at the DV midline of the wing pouch region, diffuses, and forms a concentration gradient [15]. Wg diffuses about ten cells and induces Wg effector genes such as *distal-less* (*dll*) and *vestigial* (*vg*) depending on the concentration of Wg [15–17].

NRT-Wg, an artificial membrane-tethered protein generated by the fusion of the transmembrane domain of Neurotactin and Wg, has been assumed not secreted [18]. Surprisingly, *wg [KO, NRT-Wg]* flies that have *NRT-wg* gene in place of wild-type *wg* gene develop normally, so authors claimed that the secretion of Wg is not necessary for Wg signaling and cells exposed to Wg in earlier development have cellular memory for the later development [19]. However, *wg[KO, NRT-Wg]* flies have defects in cell fate specification and proliferation, which lead to delayed organ development, malformed intestine, and sterility [19, 20]. Therefore, NRT-Wg cannot fully replace Wg for Wg signaling, and it is necessary to clarify differences between Wg and NRT-Wg.

Another factor to consider for Wg gradient is the stability of extracellular Wg. A hypothetical Wg-cleaving protease

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was proposed to explain the steepness of Wg gradient at the DV midline in a mathematical model [21]. A candidate for such protease is Sona belonged to a disintegrin and metalloprotease with thrombospondin motif (ADAMTS) family. Sona generates N-terminal domain (NTD) and C-terminal domain (CTD) by cleaving two sites in the linker region of Wg, and the epitope of the well-known anti-Wg antibody 4D4 is present in between the two cleavage sites [22]. Wg-NTD required for secretion of Wg is homologous to a class of lipid-interacting proteins whereas Wg-CTD is homologous to cytokine groups that activate cellular signaling [23–25]. Transient expression of Wg-CTD but not Wg-NTD in flies induces Wg signaling, demonstrating that Wg-CTD has Wg signaling activity. However, Wg-CTD is more specialized for cell proliferation than full-length Wg, and is less stable than full-length Wg [22]. Spatiotemporal regulation of the ratio between the two forms of Wg by Sona may be essential for fly development.

Wnts are secreted by both conventional Golgi transport and exosome-mediated secretion pathways, but it is still largely unknown how these differentially secreted Wnts contribute to the formation of Wnt gradient [26–28]. Exosomes, a major EV population with about 100 nm diameter, are intraluminal vesicles in the multivesicular body (MVB) that are released to extracellular space by the fusion of MVB with the plasma membrane [29, 30]. We recently reported a new population of EVs that are smaller than exosomes [31]. Both exosomes and this small EV group contain the EV marker CD63, but another EV marker CD81 is present only in exosomes. Furthermore, exosomes enhance their own secretion while the other group promotes cell proliferation [31].

We studied both diffusion and stability of Wg and NRT-Wg to understand the extracellular Wg gradient, and found a few important features. Firstly, Wg is secreted by both Golgi transport and via EVs whereas NRT-Wg is secreted only via EVs as a membrane-bound form. Secondly, Wg secreted by Golgi transport diffuses about ten cells but Wg-containing EVs diffuse only one cell distance. Thirdly, Sona contributes to form a steep Wg gradient. Lastly, Wg expressed in the blade region is important for wing growth and development.

Results

Extracellular Wg/Wnts are present in soluble multi-protein complex, exosomes, and a new group of EVs

Proteins and EVs secreted to extracellular space in vivo are released to culture media in cell cultures, and can be fractionated from conditioned media [27, 32]. We recently reported a new fraction that contains EVs smaller than exosomes [31]. To examine whether Wg is also present in this new fraction, conditioned media from the *tub-wg S2* cell culture were obtained, precleared and centrifuged at 100,000 × *g* to yield two different fractions: a pellet fraction (P100) containing exosomes and a supernatant fraction ($SN_{\Delta1}$). This $SN_{\Delta1}$ fraction was centrifuged at 200,000 × *g*, and a P200 pellet fraction and a supernatant fraction ($SN_{\Delta2}$) were obtained [31] (Fig. 1a).

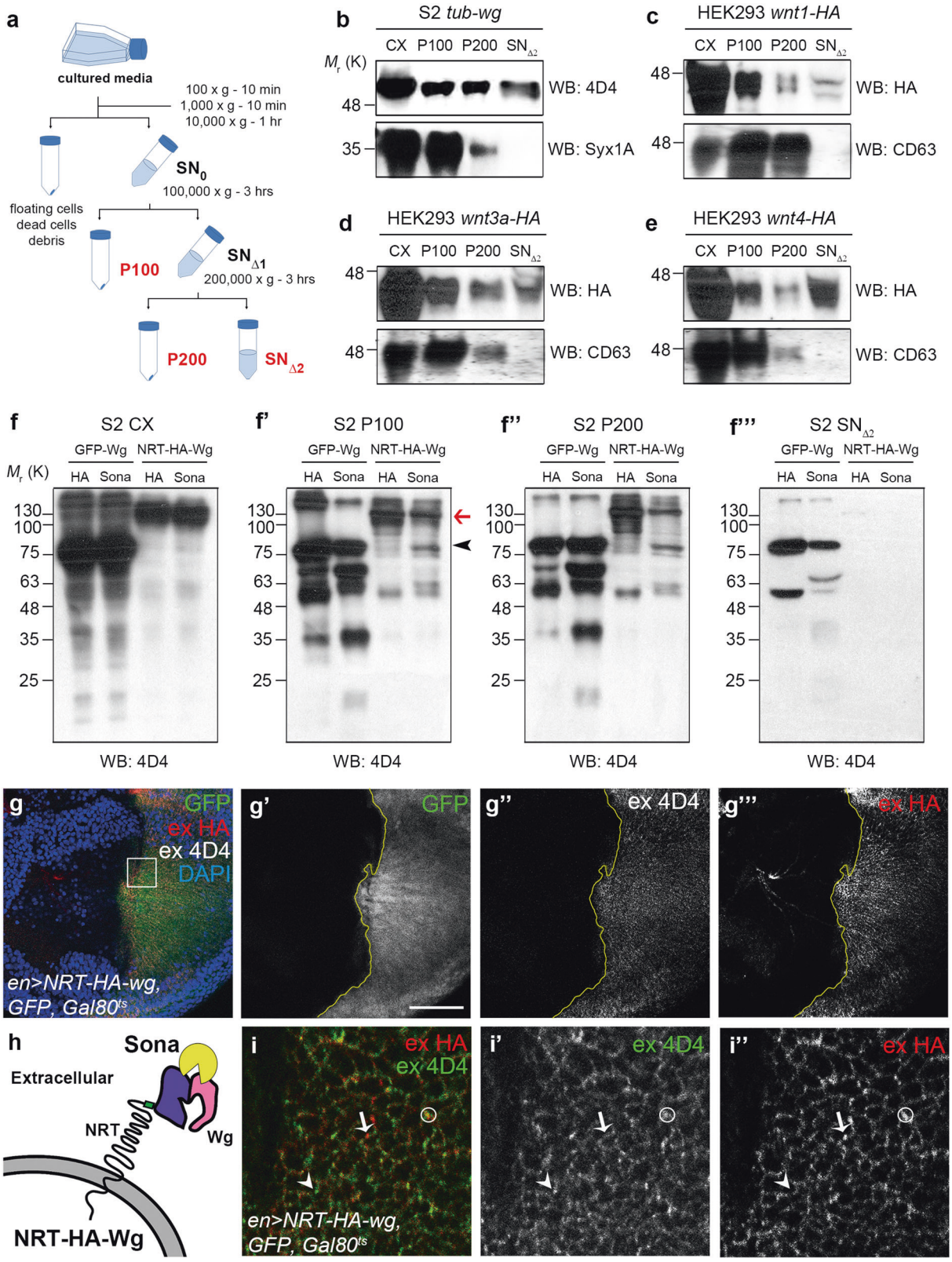
Wg was present in all fractions including the P200 fraction (Fig. 1b). Syntaxin 1A, a fly exosome marker, and CD63, a mammalian exosome marker, were present in both P100 and P200 fractions in S2 cell culture similar to the results from mammalian cell culture (Fig. S1) [31, 33]. Human Wnt1, Wnt3a, and Wnt4 were also present in all three fractions with different proportions: Wnt1 and Wnt3a are predominantly present in P100 EVs whereas Wnt4 is mainly present in $SN_{\Delta2}$ fraction (Fig. 1c–e). Therefore, both Wg and Wnts are secreted by Golgi transport and via EVs.

NRT-Wg is present in both P100 and P200 EVs

NRT-HA-Wg has a transmembrane domain of NRT, so theoretically it should be secreted as a membrane-bound form on EVs [34, 35] (Fig. 1h). To test this, the fractions were prepared from conditioned media of *S2* cells transfected with *NRT-HA-wg* cDNA (Materials and Methods). GFP-Wg as a positive control was detected in all three fractions whereas ~140 kDa NRT-HA-Wg was detected in P100 and P200 but not in $SN_{\Delta2}$ fraction (Fig. 1f'-f'''). Sona cleaved GFP-Wg in P100, P200, and $SN_{\Delta2}$ fractions, indicating that Sona cleaves Wg regardless of secretion routes. Sona also cleaved NRT-HA-Wg in both P100 and P200 fractions, and generated a fragment corresponding to Wg-CTD (Figs. 1f-f'' and S2) [22]. Furthermore, extracellular HA^+ $4D4^-$ (NRT-NTD-L1 like fragments) and HA^- $4D4^+$ (CTD-L1 like fragments) puncta that are cleaved products of NRT-HA-Wg were detected in *en > NRT-HA-wg, GFP, Gal80^{ts}* wing discs (Figs. 1g–i and S3). Therefore, NRT-HA-Wg tethered to EVs are secreted and cleaved by Sona.

EVs do not diffuse in wing discs

We then compared the patterns of Wg and NRT-Wg in *Canton-S* (*CS*) and *wg[KO, NRT-Wg]* wing discs, respectively. Both apical and basal extracellular Wg in *CS* discs was more broadly distributed than extracellular NRT-Wg in *wg[KO, NRT-Wg]* discs (Figs. 2a–d and S4), consistent with Chaudhary et al. [36]. NRT-Wg was localized mostly to the plasma membrane that may represent NRT-Wg tethered to the plasma membrane, and in some vesicular structures that may represent NRT-Wg-containing EVs.



To correlate the diffusion range of Wg and its activity, we transiently overexpressed HA-Wg and NRT-HA-Wg, and checked the expression range of Dll. As reported [18, 37–39], transiently overexpressed HA-Wg and GFP-Wg induced Dll in about ten cells from the source but NRT-HA-Wg induced Dll only at the source (Figs. 2e, f

◀ **Fig. 1 NRT-Wg is secreted via EVs and processed by Sona. a** A scheme for biochemical fractions from cultured media. **b** Western analysis for full-length Wg in various fractions. Full-length Wg from *S2 tub-wg* cell culture was detected with the anti-4D4 (Wg) antibody. An exosome marker, Syntaxin 1A (Syx1A), was detected in both P100 and P200 fraction. **c–e** Human Wnts in various fractions. *wnt1-HA, wnt3a-HA* and *wnt4-HA* cDNA constructs were expressed in HEK 293 cells. All Wnts were detected with anti-HA antibody. Both P100 and P200 fractions were confirmed by anti-CD63 antibody. **f** Various fractions from culture of S2 cells expressing *NRT-HA-wg* and *sona-HA* cDNAs are analyzed. GFP-Wg was used as a positive control for cleavage by Sona [22]. Full-length NRT-HA-Wg and a cleaved fragment generated by Sona are marked by a red arrow and a black arrowhead, respectively. **g–i** Images of extracellular NRT-HA-Wg taken at the basal region of the wing disc from the late 3rd instar larvae. NRT-HA-Wg was transiently expressed for 18 h in *en > NRT-HA-wg, GFP, Gal80^{ts}* larvae in (**g**). The boxed region in (**g**) was magnified in (**i**). Diagram of NRT-HA-Wg on EVs. NRT-HA-Wg has a HA tag between transmembrane domain of NRT and Wg [18] in (**h**). Extracellular HA$^+$ 4D4$^+$, HA$^+$ 4D4$^-$, and HA$^-$ 4D4$^+$ structures are marked with a circle, an arrow, and an arrowhead, respectively in (**i**). Scale bar, 60 µm except (**i**), 6.5 µm.

and S5). Consistent with this, extracellular HA$^+$ or 4D4$^+$ structures representing NRT-HA-Wg and its cleaved products also did not diffuse in both basal and apical regions (Figs. 1g and S6). These results raised two possibilities: EVs do not diffuse in general or only NRT-Wg-containing EVs do not diffuse. To distinguish these two, we examined the distribution pattern of the EV marker, CD63-GFP [33]. In fact, GFP signals were restricted to the CD63-GFP$^+$ clone, and extracellular GFP signals were not detected in the anterior region of *en > CD63-GFP, lacZ* wing discs (Fig. 2g, h). Thus, EVs do not diffuse in wing discs.

The Wg-NTD domain may be essential for the long-range diffusion of Wg

If Wg tethered to EVs do not diffuse, Wg secreted by Golgi transport may be solely responsible for the formation of Wg gradient in wing discs. To prove this point and examine which domain of Wg is required for diffusion, we constructed two transgenes that encode Wg-NTD and Wg-CTD fragments, each containing the Wg signal peptide at the amino-terminus [22]. Both Wg-NTD and Wg-CTD fragments were present in all biochemical fractions, indicating that they are secreted by both Golgi transport and via EVs in *S2* cells (Fig. 3a, b).

Based on the secretion of both Wg-NTD and Wg-CTD fragments by Golgi transport, we expected that both fragments may diffuse in wing discs. However, Wg-NTD diffused in *en > GFP-wg-NTD* wing discs whereas Wg-CTD did not diffuse at all in *en > wg-myc-CTD* wing discs (Figs. 3c–e and S7). This result is consistent with our previous report that the level of Dll increases only inside of the Wg-CTD-expressing clones [22]. In sum, the NTD domain may be essential for the diffusion of Wg.

Sona contributes to form a steep gradient of extracellular Wg

Wg gradient is also affected by stability of Wg, which is regulated by proteolytic enzymes. Sona is one of the strong candidates because it generates unstable Wg-CTD by cleaving stable full-length Wg [22]. We visualized the patterns of extracellular Wg at the basal region of wing discs in *CS* and two *sona* mutants ($n = 7$, each) (Fig. 4a, c, e). The intensity of extracellular Wg signals visualized by 4D4 was measured and plotted to generate Wg gradient curves. Several differences were detected between control and *sona* mutants (Fig. 4b, d, f). The peaks at the DV midline were higher and the areas under the gradient curves were larger in wild-type than *sona* mutants, which may be due to the promotion of Wg secretion by Sona [40]. Most importantly, the slopes of Wg gradient in *sona* mutants were shallower ($a = 31.528$ in *sona^{13}*; $a = 22.202$ in *sona^{18}*) than wild-type ($a = 87.225$). A similar phenomenon was observed in *wg > sona RNAi* wing discs (Fig. S8). This shallow gradient in *sona* mutants may be due to increase in the half-life of full-length Wg by lack of Wg-cleaving activity of Sona, which allows more diffusion of full-length Wg.

Wg is expressed in the presumptive wing blade region

We have shown that NRT-HA-Wg is tethered to EVs and cannot diffuse (Fig. 2). Then, the question still remains how NRT-Wg can support fly development in *wg[KO, NRT-Wg]* flies. Although the long-term cellular memory by prior exposure to Wg may be an attractive explanation [19], we considered another possibility: Wg expressed in the presumptive wing blade region (‘blade’ hereafter) is important for the normal wing development of *wg[KO, NRT-Wg]* flies.

It has been well documented that *wg* is expressed in both peripodial epithelium and disc proper in early larval wing discs [41–44], which are confirmed in *sona > GFP/wg-lacZ* flies using LacZ as a marker for *wg* transcription (Fig. 5a–c). The *wg-lacZ* was heavily expressed in the anterior ventral domain and at the DV midline in wing discs of early third instar larvae (Fig. 5a, b). After the mid-third instar stage, expression of *wg* became more prominent at the DV midline and the presumptive hinge region but became weaker in the blade (Fig. 5c–e). Interestingly, other *wg* reporters, *wg-Gal4* and *wg[KO, Gal4]* [19, 45], were also expressed in the blade with similar patterns to the *wg-lacZ* reporter at a much higher level (Fig. 5e–g). Besides, a specific region near the anterior-posterior (AP) boundary did not express *wg* in all *wg* reporters (asterisks in Fig. 5a–g). In case of *sona*, it was expressed as a patchy

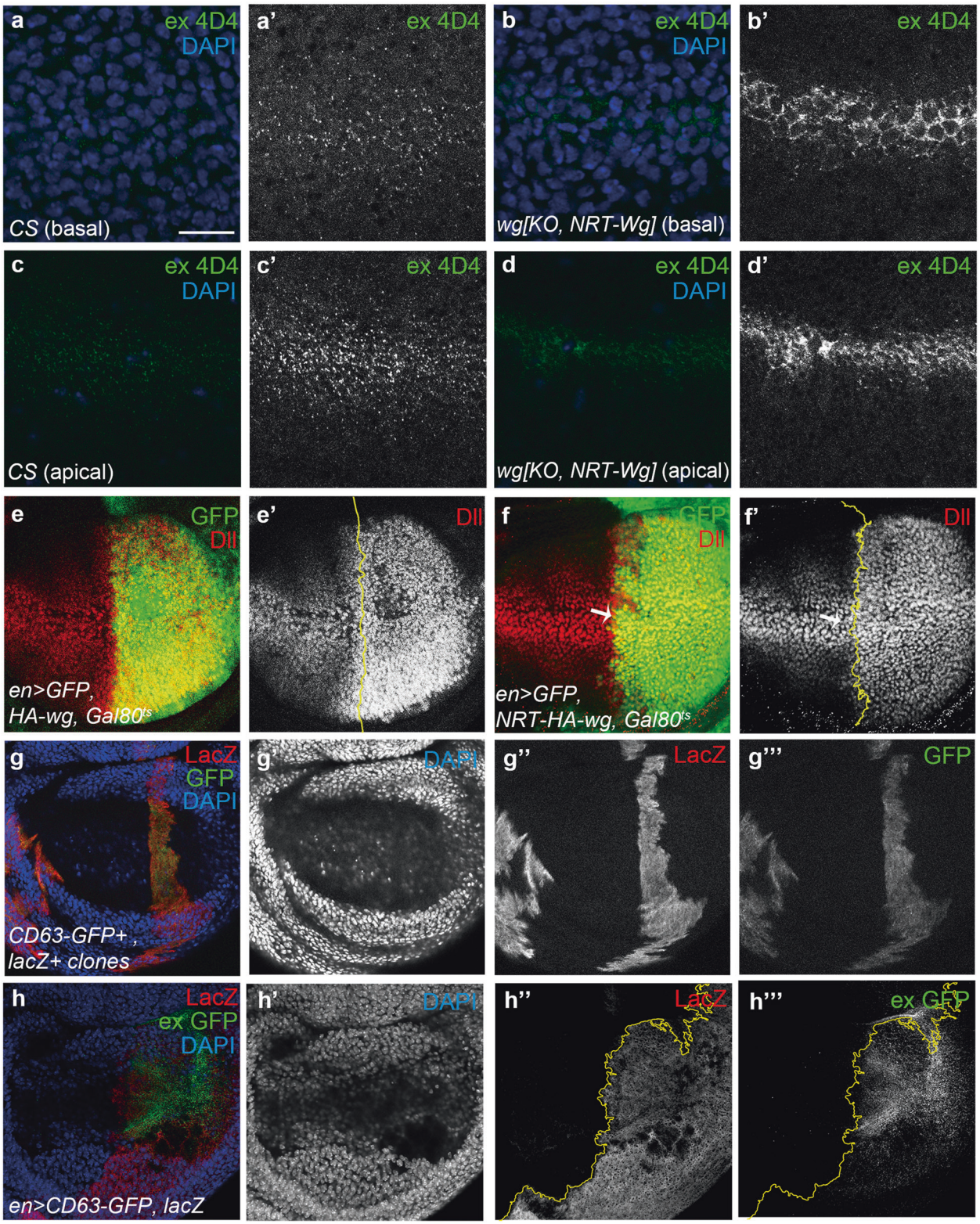


Fig. 2 NRT-Wg-containing EVs do not diffuse in wing discs. Yellow lines demarcate the anterior and the posterior boundary in (**e**), (**f**), and (**h**). **a**–**d** Distribution patterns of extracellular Wg in the DV boundary of wing discs in the late 3rd instar *CS* and *wg[KO, NRT-Wg]* larvae. Images in (**a**) and (**b**) were taken by focusing at basal extracellular matrix (ECM) while those in (**c**) and (**d**) were taken by focusing at apical ECM. **e**, **f** Both HA-Wg and NRT-HA-Wg were transiently expressed in the posterior region by *en-Gal4* driver. Dll was induced up to ten cells in the anterior region of *en > GFP, HA-wg, Gal80^{ts}* discs in (**e**). NRT-HA-Wg induced Dll cell-autonomously and in juxtaposed cells in *en > GFP, NRT-HA-wg, Gal80^{ts}* discs (white arrow) in (**f**). **g** No diffusion of GFP^{+} signals from CD63-GFP^{+} LacZ^{+} clones generated by flp-out method [67]. **h** Extracellular CD63-GFP visualized by anti-GFP antibody in the *en > CD63-GFP, lacZ* wing disc. Scale bar, (**a**), (**b**) 40 μm; (**e**–**h**) 60 μm.

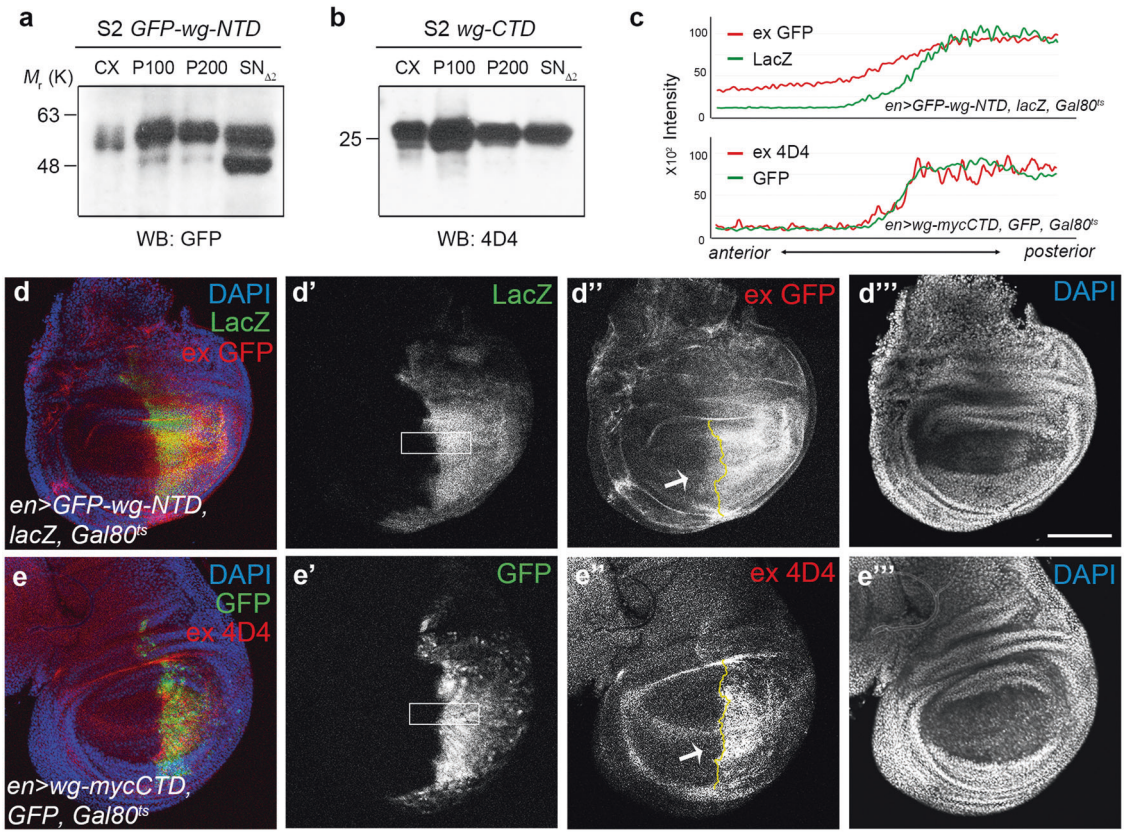


Fig. 3 Extracellular Wg-NTD diffuses whereas Wg-CTD does not diffuse. Yellow lines mark the AP boundary of wing discs in (**d**) and (**e**). **a**, **b** CX, P100, P200, and $SN_{\Delta 2}$ fractions from S2 cells expressing *GFP-wg-NTD* cDNA in (**a**) and *wg-mycCTD* cDNA in (**b**). **c**–**e** Extracellular GFP signal representing GFP-Wg-NTD at the basal ECM and LacZ signal representing the *en* region are compared in upper (**c**) and extracellular 4D4 signal representing Wg-MycCTD and GFP signal representing the *en* region are compared in lower (**c**). The plotted intensity of extracellular GFP and 4D4 signal in (**c**) were measured in the boxed regions in (**d'**) and (**e'**), respectively. Extracellular GFP signals representing GFP-Wg-NTD protein diffused (arrow in **d''**). Extracellular 4D4 signals representing Wg-CTD in (**e**) did not diffuse (arrow in **e''**). Scale bar, 100 μm.

pattern in the entire pouch during larval stages (Fig. 5a–c, h). Since Sona promotes Wg secretion as well as cleaves Wg, dynamic changes in the transcriptional patterns of *wg* and *sona* may be essential for optimizing the amount of full-length Wg and Wg-CTD in any given location of the wing disc.

To check whether the pattern of these *wg* reporters represents the transcription pattern of *wg*, a lineage analysis was performed using G-trace system with *wg-Gal4* drivers [46]. In this cell-autonomous FLP-out marking system, cells expressing a given *Gal4* at a sampling time are marked with RFP representing 'real-time' expression, while cells that have expressed the given *Gal4* during the earlier developmental time are marked with GFP representing 'lineage'. The 'real-time' RFP patterns induced by these *wg-Gal4* drivers were similar to the LacZ patterns induced by *wg-Gal4* and *wg[KO, Gal4]* drivers (Fig. 5i", j"). Besides, a region near AP boundary devoid of *wg* expression was also detected (asterisks in Fig. 5i", j"). The 'lineage' GFP signals were present in almost all disc proper cells plus anterior peripodial epithelium, consistent with the wide expression of Wg in early larval wing discs (Fig. 5i', j'). The patterns of these *wg* reporters were also fairly similar to those of Wg protein in the blade (Fig. S9). In sum, Wg expressed in the blade may be essential for the wing development of not only *wg[KO, NRT-Wg]* but also normal flies.

Patterns of *vgQE* and Delta in the wing blade correlate with that of *wg*

Pattern of *wg* in the blade suggests that the blade cells may express other genes similar or opposite pattern to *wg*, and we identified two such genes, *vestigial* (*vg*) and *Delta* (*Dl*). It has been shown that the expression of *vg* in the blade is carried out by the *vgQE*-enhancer [17, 47, 48]. Interestingly, GFP representing *wg* transcription and LacZ representing *vg* transcription in the blade of *wg-Gal4/UAS-GFP; vgQE-lacZ* wing discs were mutually exclusive (Fig. 6a, b). For instance, the region near the AP boundary had a low level of GFP but a high level of LacZ (asterisks in Fig. 6a', a"). Similar results were obtained in *wg[KO, Gal4]/UAS-GFP; vgQE-lacZ* wing discs (Fig. S10).

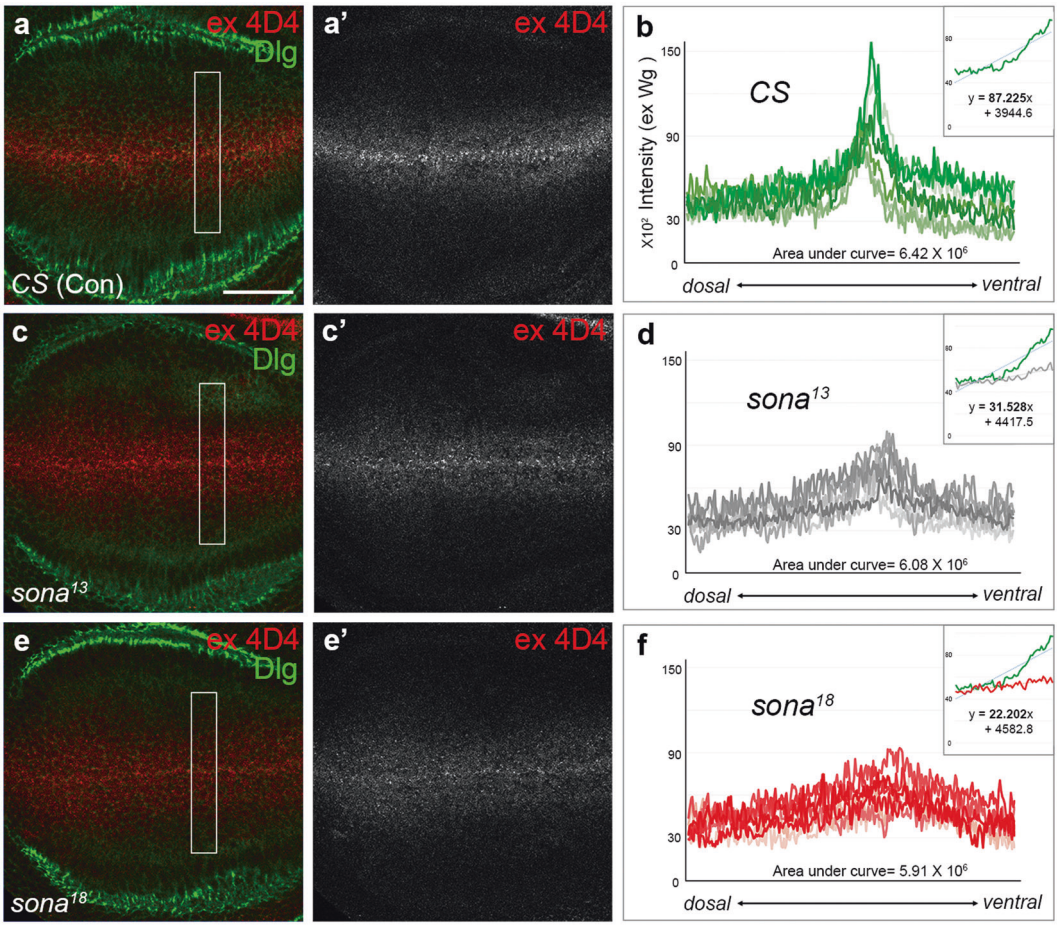


Fig. 4 Slopes of Wg gradient in *sona* wing discs are shallower than wild-type. Distribution patterns of extracellular Wg in the DV boundary of late 3rd instar wing discs in *CS* (**a**), *sona*^{13} (**c**) and *sona*^{18} (**e**) larvae. Extracellular Wg at basal ECM was detected with anti-4D4 antibody. The signal intensities of extracellular Wg in the boxed regions encompassing the dorsal and ventral wing pouch in (**a**), (**c**) and (**e**) ($n = 7$, each) are co-plotted in (**b**), (**d**) and (**f**). The average intensity of background signal was 2.09×10^2, approximately one tenth or less than the lowest intensity of all samples. The trend line was drawn by averaging all seven curves and the slope was calculated by regression analysis with the values obtained from the dorsal edge to the DV midline. Scale bar, 60 µm.

Dl is a Notch ligand acting upstream of *wg*, and regulates *wg* transcription in regions for future wing margin and veins [49]. In fact, the level of *wg-lacZ* was increased by over-expression of Dl in the blade (Fig. 6c, d). Dl was also expressed at a higher level along the future vein regions in a similar pattern to GFP in *wg > GFP* wing discs, suggesting that Dl in the blade turns on *wg* transcription (Fig. 6e, f). Thus, expression of *wg, vg*, and *Dl* in similar or mutually exclusive pattern in the blade may be crucial to specify veins and interveins as well as to modulate the rate of cell proliferation in different parts of the blade.

Discussion

It had been widely accepted for decades that secreted Wg diffuses in the extracellular space, forms a concentration gradient, and induces gene expression in a concentration-dependent manner. Here, we report that Wg tethered to EVs does not diffuse and induces only a short-range Wg signaling in autocrine and juxtacrine manners (Fig. 7a). In contrast, soluble Wg in a multi-protein complex secreted by conventional Golgi transport diffuses, forms a gradient, and induces a long-range signaling (Fig. 7b). Furthermore, Sona contributes to the formation of steep Wg gradient in wing discs because it cleaves stable Wg and generates unstable Wg-CTD.

The secretion and diffusion of Wg tethered to EVs

EVs including exosomes have recently drawn a lot of attention because of their signaling activity and medical application [50, 51]. We found that Wg/Wnts are present in both P100 and P200 EV fractions (Fig. 1). These P100 and P200 fractions promote exosome production and cell proliferation, respectively [31]. In addition to Wnt activity as

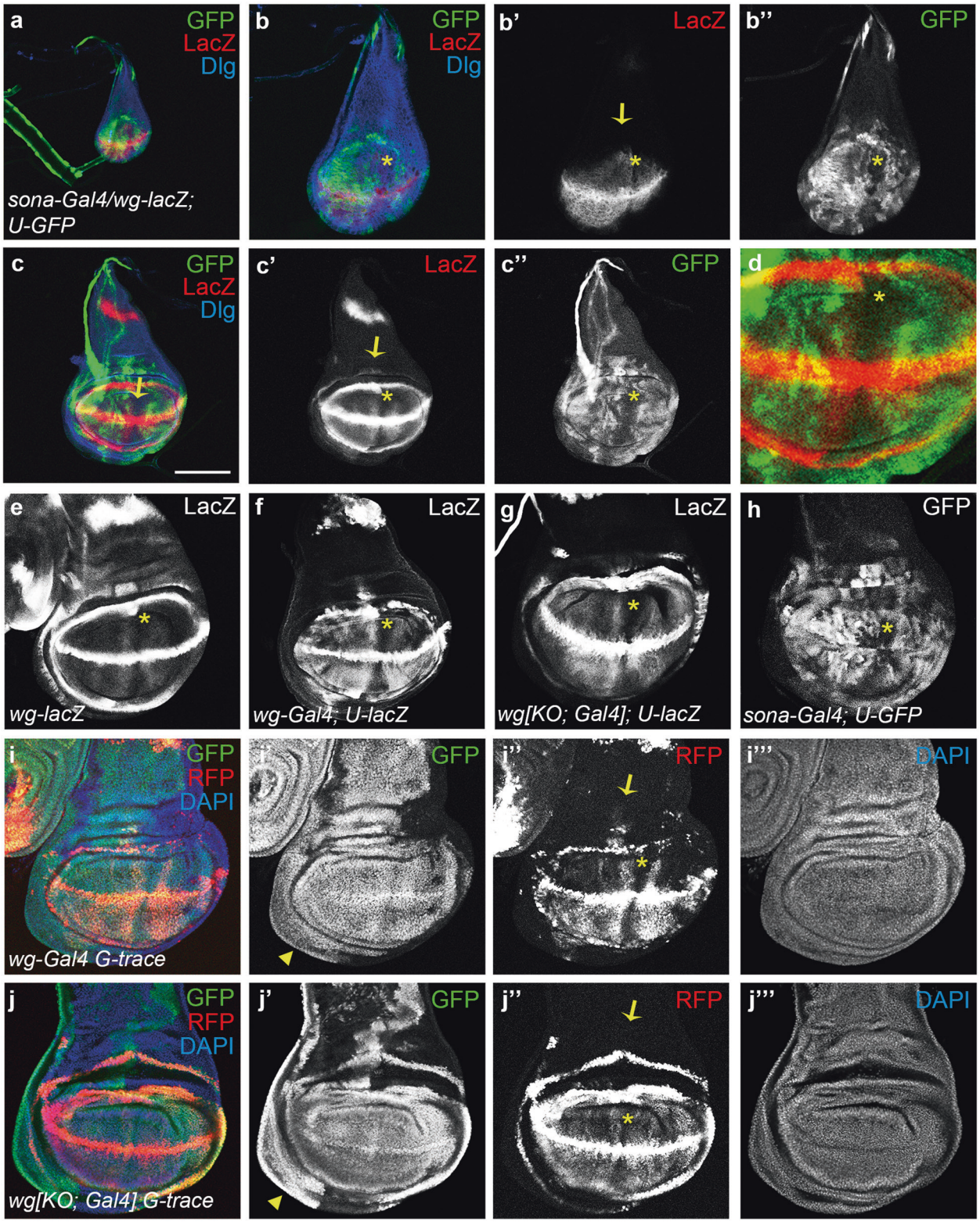


Fig. 5 Transcription patterns of *wg* and *sona* are patchy in the wing blade. Yellow asterisks mark specific regions near the AP boundary where neither *wg* nor *sona* is expressed. Yellow arrows indicate absence of *wg* transcription in all *wg* enhancers examined. **a**, **b** Transcriptional pattern of *wg* and *sona* in *sona > GFP, wg-lacZ* wing discs of the late 2nd instar larvae. **a** is magnified five times in (**b**). **c**, **d** The transcriptional pattern of *wg* and *sona* in mid 3rd instar wing discs. **c** is magnified nine times in (**d**). **e**–**g** LacZ pattern of three *wg* reporters in late 3rd instar wing discs: *wg-lacZ* (**e**), *wg-Gal4 > lacZ* (**f**) and *wg[KO; Gal4]>lacZ* (**g**). **h** GFP pattern in *sona > GFP* late 3rd instar wing discs. **i**, **j** G-trace analysis for *wg-Gal4* and *wg[KO; Gal4]* drivers in late 3rd instar wing discs. RFP signals represent the real-time expression of the driver, and GFP signals represent cell lineage that had expressed the driver during earlier time of development. Scale bar, 100 μm except (**b**), 50 μm.

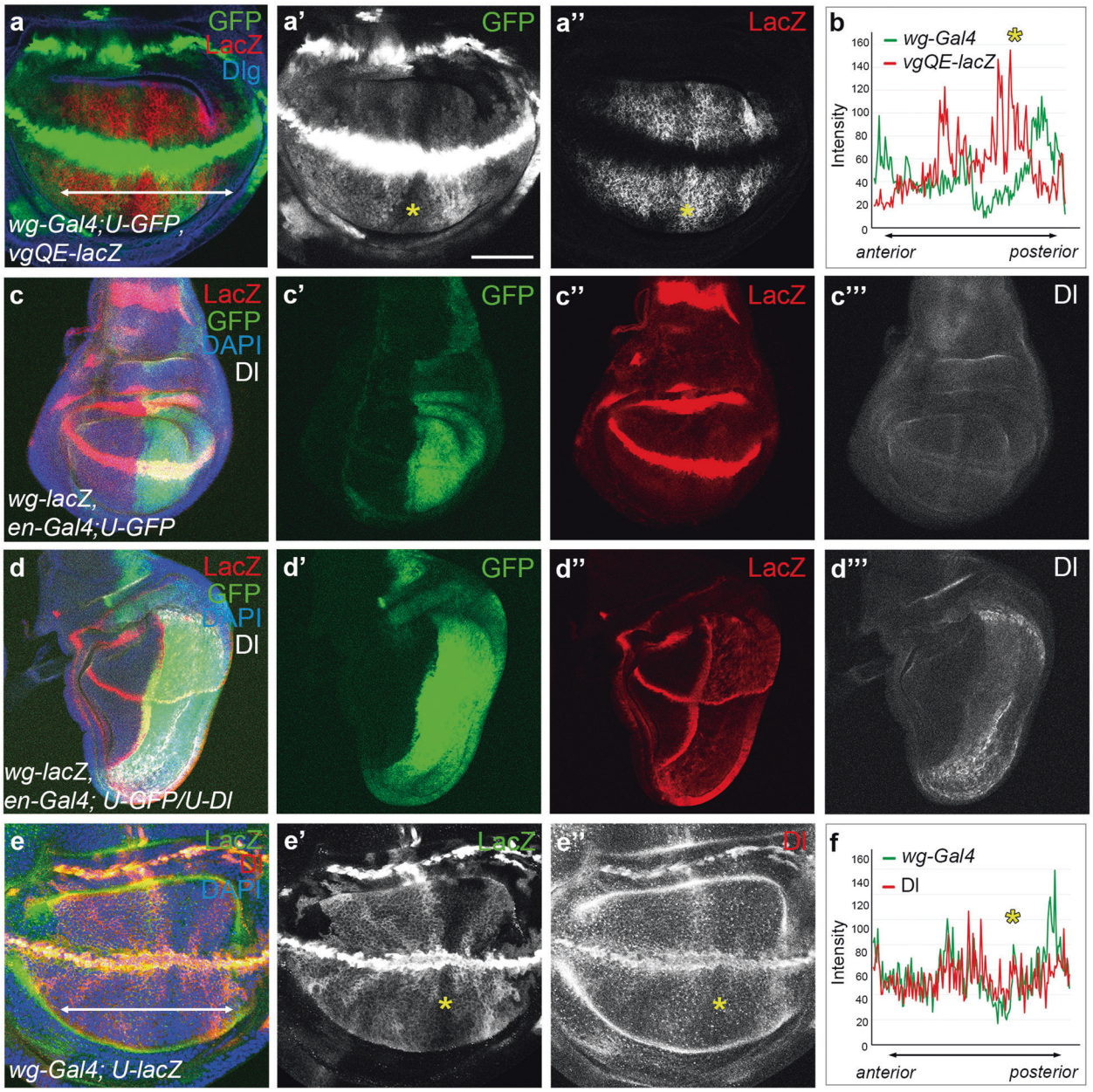


Fig. 6 The pattern of *wg* in the blade is opposite to *vgQE*, but similar to Dl. The specific region near the AP boundary is marked with yellow asterisks. **a**, **b** GFP and LacZ patterns in *wg > GFP; vgQE-lacZ* wing disc (**a**) and the black and white images (**a'**, **a''**). Intensities of red and green signals along the white-line in (**a**) are plotted in (**b**). **c**, **d** As a control, Wg-lacZ and Dl patterns in *wg-lacZ/en-Gal4; UAS-GFP* wing discs are shown (**c**). Overexpression of Dl induced the LacZ expression in the posterior region of *wg-lacZ/en-Gal4; UAS-GFP, UAS-Dl* wing discs (**d**). **e**, **f** LacZ and Dl patterns in *wg > lacZ* wing discs (**e**). Intensities of red and green signals on the white-line in (**e**) are plotted in (**f**). Scale bar, (**a**), (**e**) 60 μm; (**c**), (**d**) 100 μm.

mitogens [17, 18, 52], Wnt3a and Wnt5b promote exosome production in primary cultured microglia and melanoma cells, respectively [53, 54]. Therefore, Wnts on EVs may be one of the key proteins that promote cell proliferation and exosome production in a juxtacrine manner.

Wnt1 and Wnt3a were abundantly present especially in the P100 fraction whereas Wnt4 was abundantly present in the $SN_{\Delta 2}$ fraction (Fig. 1). Therefore, Wnt1 and Wnt3 may be preferentially secreted as tethered forms to EVs and act in a juxtacrine manner. Consistent with this speculation, Wnt3a expressed from Paneth cells induces a short-range Wnt signaling in the intestinal stem cell niche [55] and the majority of Wnt3a is secreted as a membrane-associated form in MDCK cell culture [56]. These Wnt/Wg secreted by multiple secretion routes may play distinct roles in different tissues. Since glycosyl groups in Wnts determine by which pathway Wnts are secreted [56], it will be interesting to find the potential link between glycosyl groups and the secretion

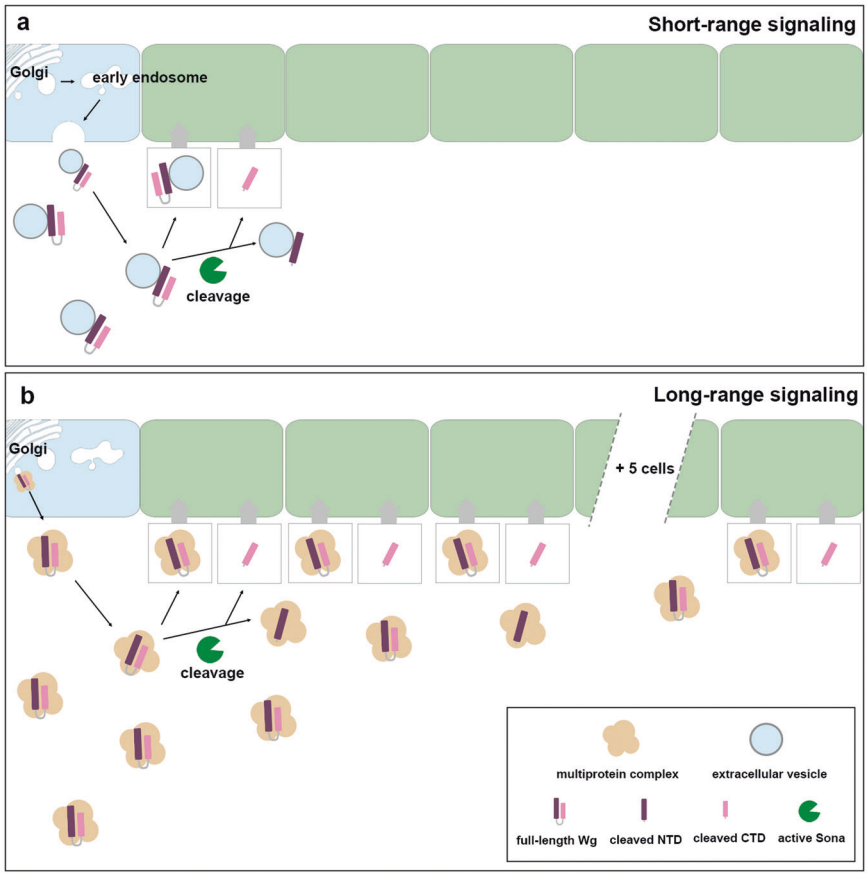


Fig. 7 Wg containing EVs induces the short-range Wg signaling whereas Wg in a multi-protein complex induces the long-range signaling. a A model to explain the behavior of Wg secreted by EVs. Wg-containing EVs are channeled into two pathways. (1) When Sona is absent, Wg-containing EVs bind to the juxtaposed cells and induce Wg signaling. (2) When Sona is present, Wg is cleaved to Wg-CTD, Wg-CTD then binds to the juxtaposed cells and induces Wg signaling. **b** A model to explain the behavior of Wg secreted by conventional Golgi transport in two different pathways. (1) When Sona is absent, Wg^{FL} in multi-protein complex diffuses about 10 cells, forms Wg gradient and induces the long-range signaling. (2) When Sona is present, Wg^{FL} in the multi-protein complex is cleaved by Sona, Wg-CTD binds to cells nearby and induces Wg signaling. The distance that Wg^{FL} travels depends on the level of Sona.

route of Wg in various fly tissues. Moreover, NRT-Wg signaling to the Frizzled 2 (Fz2) rather than the Fz1 receptor plays an important role in cell viability [36]. Thus, each Fz receptor may exhibit differential binding affinity for Wg in multi-protein complex, Wg on EVs, or Wg-CTD.

Wing vs. other organ defects in *wg[KO, NRT-Wg]* flies

wg[KO, NRT-Wg] flies that lack Wg secreted by Golgi transport have normal wing margin bristles, so NRT-Wg on EVs seems to be fully responsible for the differentiation of wing margin bristles. However, the size of their wings is about 10–12% smaller than wild-type wings [19], suggesting that the diffusion of Wg secreted by Golgi transport is required for wing growth to about 10–12%, and Wg on EVs expressed in the blade is mainly responsible for wing growth of *wg[KO, NRT-Wg]* flies. Based on these data on *wg[KO, NRT-Wg]* flies, one can speculate that Wg on EVs plays more significant roles for wing growth and development than Wg secreted by Golgi transport in wild-type flies.

Although wings of *wg[KO, NRT-Wg]* flies are almost normal, these flies show defects in cell proliferation and fate specification during gut development [20]. *wg[KO, NRT-Wg]* flies also show severe defects in proximo-distal patterning of Malphigian/ureter tubules and neuronal specification in the visual system [57, 58]. Thus, Wg secreted by Golgi transport and formation of Wg gradient seem to play more significant roles than Wg on EVs in these aforementioned organs compared to wings.

Wg-cleaving proteases in the formation of Wg gradient

The clear difference between the slope of Wg gradient in *sona* and wild-type discs demonstrates that Sona affects Wg gradient. The peak level of extracellular Wg at the DV midline of *sona* wing discs was lower than that in wild-type, which is consistent with the involvement of Sona in Wg secretion (Fig. 4) [22]. To interpret the slope of Wg gradient, it is necessary to consider that Wg signals detected by the 4D4 antibody are mostly from full-length Wg and Wg-NTD but little from unstable Wg-CTD [22]. The steep Wg gradient in wild-type wing discs indicates that only a small fraction of full-length Wg is diffused far from the DV midline probably because Sona efficiently cleaves full-length Wg. By the same token, the shallow Wg gradient in *sona* wing discs indicates that full-length Wg is increased in its level by lack of Sona activity, and diffuses farther.

Tiki is another Wnt-cleaving metalloprotease identified in *Xenopus*, and its homologs are found in vertebrates but

not in flies [59, 60]. Tiki cuts the N-terminal residues of several Wnts, which minimizes receptor-binding capacity and reduces the solubility of extracellular Wnt [61, 62]. Whether Tiki affects Wnt gradient has not yet been reported. It is possible that other Wg-cleaving proteases besides Sona may exist and affect Wg signaling in flies because cleaved products of Wg are repeatedly detected in the absence of Sona in S2 cell culture [22].

Distinct expression patterns of Wg and Sona in the wing blade

It has been widely accepted that Wg secreted from the cells in the DV midline of the wing discs is responsible for the growth and differentiation of the entire wing. We and others have shown that Wg is expressed not only in the DV midline but also in the blade region. Wg is heavily expressed in the ventral region of wing discs during the early larval stage [41–44] (Fig. 5). We showed that *wg* is also expressed in the blade during the late third instar stage. Furthermore, *wg* is heavily expressed in all veins of adult wings [63], which is consistent with our finding that pattern of Wg is similar to that of Dl in the blade region of the wing disc in the late 3rd larval stage (Fig. 6).

Simple diffusion of Wg from the DV midline to the entire blade may not be adequate to finely control the activity of Wg signaling in the local environment. In line with this, *wg* and *sona* are expressed in patchy patterns in the blade (Fig. 5), which may allow modulation of the amount of full-length Wg and Wg-CTD at any given location. Such fine-tuning of full-length Wg vs. Wg-CTD may be essential for coordinating rates of cell proliferation and patterning [22]. We propose that Wg tethered to EVs is secreted from cells at the DV midline to initiate neuronal differentiation for the formation of wing margin bristles by the short-range signaling, whereas Wg secreted by Golgi transport diffuses to form Wg gradient and support the growth of cells about ten cells from the DV midline. Most importantly, the low amount of Wg secreted from the cells in the blade via EVs play important roles in growth and patterning of structures such as veins and interveins. It will be interesting to find out whether these findings on fly Wg are relevant to Wnts for the development and growth of other animals.

Materials and methods

Drosophila strains, transgenic lines, and DNA constructs

UAS-wg-mycCTD, UAS-GFP-wg-NTD, sona^{13}, and *sona*^{18} flies are described elsewhere [22, 40]. *UAS-CD63-GFP* [33], *UAS-GFP-wg* [64], *wg[KO; Gal4], wg[KO, NRT-Wg]* [65], *UAS-NRT-HA-Wg* [18], *wg-Gal4* [45], and *vgQE-lacZ* [66] were kindly provided from other labs. All other fly lines were obtained from the Bloomington stock center. *Wnt* cDNAs were obtained from J. W. Kim. *pUAST-CD63-GFP* was constructed with *CD63-GFP* cDNA in *CD63-pEGFP C2* (Addgene). The *pUAST-NRT-HA-wg* was constructed by recombining the *pUAST* vector with *NRT* cDNA (*WG35*) and *HA(flu)-wg* cDNA (*WG143*). These *WG35* and *WG143* constructs were kindly provided by G. Struhl.

Cell lines, cell culture, and exosome preparation

Drosophila S2 *tub-wg* and S2 cell lines were obtained from DGRC. *Drosophila* S2 cell were grown in M3 media (Sigma-Aldrich) supplemented with 10% IMS (Sigma-Aldrich) at 25 °C. Stable cell lines were grown with hygromycin in 10% IMS M3 media, and S2 *tub-wg* cells were cultured in 10% FBS M3 media. Transfections were carried out with Cellfectin (Invitrogen) according to the manufacturer's instructions. For the preparation of EVs (P100, P200), 7–40 ml of conditioned media obtained from cultures (1.25×10^6 cells/ml) were used as described [31]. Human HEK293 cells obtained from KCBL were grown in RPMI 1640 media (Gibco) supplemented with 10% FBS (Gibco) at 37 °C with 5% CO_2.

Immunocytochemistry and Western analysis

Fly larvae were cultured at 25 °C unless stated otherwise. Wing discs from the late third instar larvae were used for intracellular staining and extracellular staining [15]. For immunocytochemistry, we used 1:100; 4D4 (DSHB, mouse), 1:200; HA (Roche, rat), 1:200; GFP (Abcam, rabbit), 1:100; Dl (DSHB, mouse), 1:100; Dll (Santa Cruz, goat). For extracellular staining, we used ten times more antibodies than for intracellular staining. Fluorescent images were captured using a Zeiss LSM laser scanning confocal microscope and processed with Adobe Photoshop. Plot analysis and intensity measurements were performed on raw data processed with Zen 2012 and ImageJ under identical conditions. Western analysis was carried out as described [40]. For western analysis, we used 1:5,000; HA (Santa Cruz, rabbit), 1:1,000; HA (Roche, rat), 1:1,000; Wg (DSHB, mouse), 1:1,000; CD63 (SBI, rabbit), 1:1,000; CD63 (Santa Cruz, mouse), 1:1,000; GFP (Abcam, rabbit), and 1:1,000; Syx1A (DSHB, mouse).

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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