The EMBO Journal (2008), 1–13 | © 2008 European Molecular Biology Organization | Some Rights Reserved 0261-4189/08
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THE EMBO JOURNAL

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Reggie-1/flotillin-2 promotes secretion of the long-range signalling forms of Wingless and Hedgehog in *Drosophila*

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The lipid-modified morphogens Wnt and Hedgehog diffuse poorly in isolation yet can spread over long distances *in vivo*, predicting existence of two distinct forms of these mophogens. The first is poorly mobile and activates short-range target genes. The second is specifically packed for efficient spreading to induce long-range targets. Subcellular mechanisms involved in the discriminative secretion of these two forms remain elusive. Wnt and Hedgehog can associate with membrane microdomains, but the function of this association was unknown. Here we show that a major protein component of membrane microdomains, reggie-1/flotillin-2, plays important roles in secretion and spreading of Wnt and Hedgehog in *Drosophila*. Reggie-1 loss-of-function results in reduced spreading of the morphogens, while its overexpression stimulates secretion of Wnt and Hedgehog and expands their diffusion. The resulting changes in the morphogen gradients differently affect the short- and long-range targets. In its action reggie-1 appears specific for Wnt and Hedgehog. These data suggest that reggie-1 is an important component of the Wnt and Hedgehog secretion pathway dedicated to formation of the mobile pool of these morphogens.

The EMBO Journal advance online publication, 24 January 2008; doi:10.1038/sj.emboj.7601981
Subject Categories: membranes & transport; development
Keywords: hedgehog; morphogen; reggie/flotillin; secretion; wingless

Introduction

Morphogens are molecules spreading from the region of production and inducing tissue patterning in a concentration-dependent manner (Lawrence, 2001; Lander, 2007). Different threshold levels of the morphogen concentrations exist, which through appropriate levels of receptor activation are translated into transcriptional induction of different target genes.

Examples of morphogens include the families of Wnt, Hh (Hedgehog) and TGFbeta-secreted proteins, which can bear multiple post-translational modifications. Wnt proteins are doubly *N*-glycosylated (Tanaka *et al*, 2002) and bear palmitate (Willert *et al*, 2003) and palmitoylate (Takada *et al*, 2006) groups. Hh is palmitoylated and cholesterol-modified (Porter *et al*, 1996; Pepinsky *et al*, 1998). These modifications are necessary for the proper secretion and activity of the morphogens (Miura and Treisman, 2006), and require specific mechanisms for Wnt and Hh secretion and spreading. Multipass transmembrane proteins Wntless/Evi and Dispatched are indispensable for secretion of Wnt and Hh, respectively (Burke *et al*, 1999; Banziger *et al*, 2006; Bartscherer *et al*, 2006). Due to the hydrophobic nature of the modifications, secreted Wnt and Hh are not freely diffusible in their isolated form. In aqueous solutions, Wnt proteins aggregate and precipitate (Willert *et al*, 2003). *In vivo*, Wnt and Hh bind to the cell surface and extracellular matrix, which strongly reduces their diffusion properties (Papkoff and Schryver, 1990; Lee *et al*, 1994).

How is effective long-range diffusion of Wnt and Hh achieved *in vivo*? It has been speculated that two routes for their secretion exist (Coudreuse and Korswagen, 2006; Hausmann *et al*, 2007). The first would involve a 'default' secretion pathway, releasing monomeric or low-degree oligomeric forms of the morphogens, whose diffusion would be very limited. Accumulation of these forms close to the source of production turns on short-range target genes. The second route, poorly understood, is predicted to produce the morphogens specifically packed for efficient spreading permitting induction of the long-range targets. Multiple ways to pack morphogens for long-range diffusion may exist. In *Drosophila*, Wnt and Hh can associate with fat body-derived lipoprotein particles, promoting spreading and activation of long-range target genes (Panakova *et al*, 2005). In cell culture, mammalian Hh can form highly diffusive, biologically active aggregates of a high molecular weight (Zeng *et al*, 2001).

It remains elusive what kind of intracellular mechanism can direct the morphogens into the long diffusion range-specific secretion, as opposed to the default secretion route. Wnt and Hh can associate with the detergent-resistant membrane (DRM) microdomains (Rietveld *et al*, 1999; Zhai *et al*, 2004), which has been proposed important for Hh packing into high-order aggregates (Zeng *et al*, 2001); lipid modifications of Wnt and Hh are required for the DRM association.

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Received: 1 July 2007; accepted: 19 December 2007

Membrane microdomains are involved in a variety of cellular functions, for example, being platforms for specific signal transduction cascades, as well as for particular types of intracellular trafficking (Pike, 2004; Le Roy and Wrana, 2005). Different types of microdomains can be described by their major resident proteins, such as annexin, reggie/flotillin, or caveolin (absent in *Drosophila*) (Le Roy and Wrana, 2005).

In this article, we show that a major component of the membrane microdomains, reggie-1/flotillin-2 (Langhorst *et al*, 2005; Stuermer and Plattner, 2005), plays important roles in Wnt and Hh secretion in *Drosophila*. Overexpression of reggie-1 leads to increased secretion and enhanced spreading of these morphogens. Loss of function of reggie-1 results in the reciprocal reduction in the morphogen gradient. Our results suggest that reggie-1 is an integral part of the intracellular trafficking events directing Wnt and Hh morphogens along the secretion route destined for the long-range spreading.

Results

Overexpression of reggie-1 in Drosophila wing results in loss of a subset of Wnt-signalling responses

Two reggie isoforms (reggie-1/flotillin-2 and reggie-2/flotillin-1) exist in mammals and fruit flies (Hoehne *et al*, 2005; Langhorst *et al*, 2005). Co-overexpression of both isoforms in *Drosophila* results in massive morphological defects, whereas reggie-2 alone produces no phenotypes (Hoehne *et al*, 2005). To investigate whether reggies affect Wg (Wingless, *Drosophila* Wnt-1) signal transduction, we repeated the overexpression in a more controlled manner using the *MS1096-Gal4*, *engrailed-Gal4* (*en-Gal4*), *dpp-Gal4* or *tubulin-Gal4* (*tub-Gal4*) lines. As we obtained identical initial results by expression of reggie-1 alone or coexpression of reggie-1 + 2, experiments presented in this paper describe expression of reggie-1 alone.

Adult wings lost wing margin structures in the regions of reggie-1 overexpression (Figure 1B and C). Since such phenotypes can arise from defective Wg signalling, we analysed the expression of Wg target genes in wing imaginal discs of late third instar larvae. We found downregulation of the short-range Wg target genes Cut and Senseless (Sens) in the reggie-1 overexpression domain (Figure 1D and E; Figure 2C; Figure 1F shows wild-type Sens expression). Another readout for high Wg signalling is the zone of non-proliferating cells (ZNC) forming close to the source of Wg synthesis (Johnston and Edgar, 1998; Figure 1F). The ZNC was eliminated in the region of reggie-1 overexpression (Figure 1G). Similar effect can be obtained by expression of Axin (an inhibitor of Wg signalling) or cyclin E (which forces exit from the cell-cycle arrest; Johnston and Edgar, 1998). However, unlike Axin and reggie-1, cyclin E was unable to downregulate Sens (Supplementary Figure S1). Thus, analysis of responses to high levels of Wg signalling revealed that overexpression of reggie-1 reduced Wg signalling.

Loss of Wg signalling can result in misexpression of the hinge marker Homothorax in the wing pouch (Azpiazu and Morata, 2000). Overexpression of reggie-1 reduced the size of the pouch (but not hinge) domain in an autonomous manner (Figure 1C; also compare Figure 2A and B). As Wg signalling controls growth (Day and Lawrence, 2000; Johnston and Gallant, 2002), this reduction in pouch size could be attributed to a defective Wg signalling. However, pouch

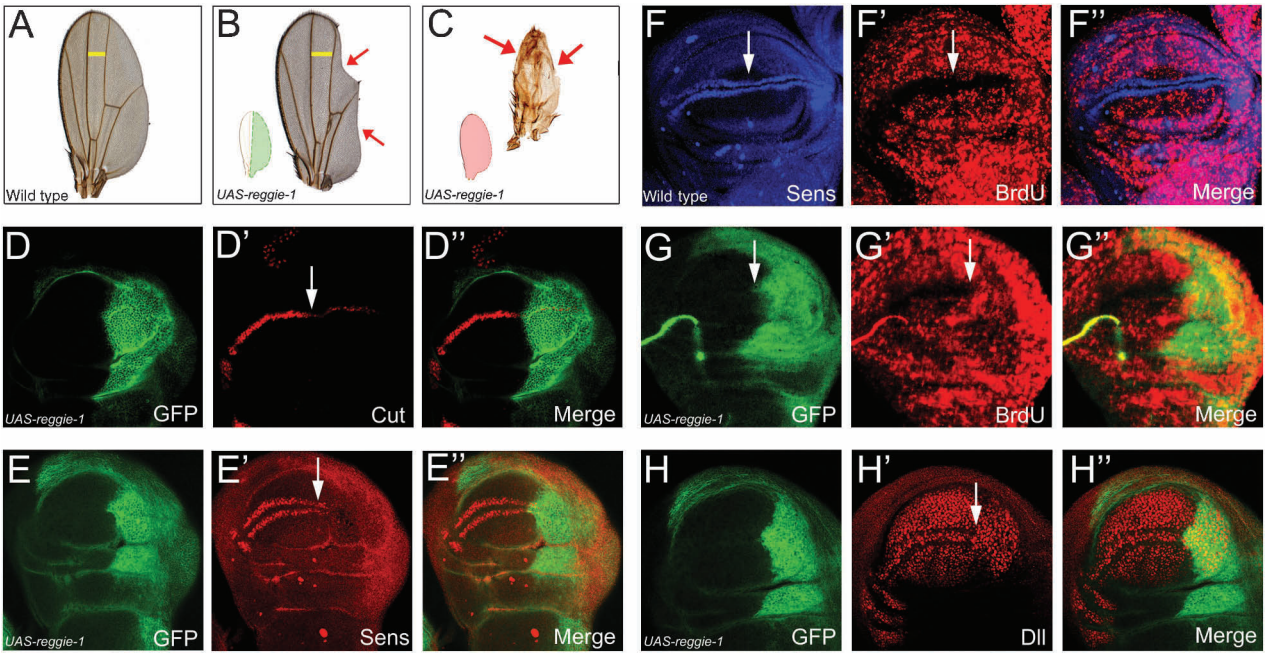

Figure 1 Overexpression of reggie-1 leads to loss of a subset of Wg responses. (**A**) Wild-type adult wing. (**B**, **C**) Adult wings overexpressing reggie-1 by *en-Gal4* (B) or *MS1096-Gal4* (C) lose wing margin (arrows); inserts show where these lines drive expression. Panel B also shows an Hh phenotype: broadening between veins 3 and 4 (yellow bar). (**D**–**H**) Wing imaginal discs of late third instar larvae. Ventral is up, posterior is right. White arrows indicate the A/P border; *en-Gal4* expresses to the right from the arrows, marked by GFP. *en-Gal4*-driven reggie-1 downregulates Wg short-range targets Cut (D′) and Sens (E′), but not the long-range target Dll (**H**′). Wild-type Sens is shown in panel F. The ZNC visualized by the gap in BrdU staining (F′) is autonomously eliminated by reggie-1 (G′).

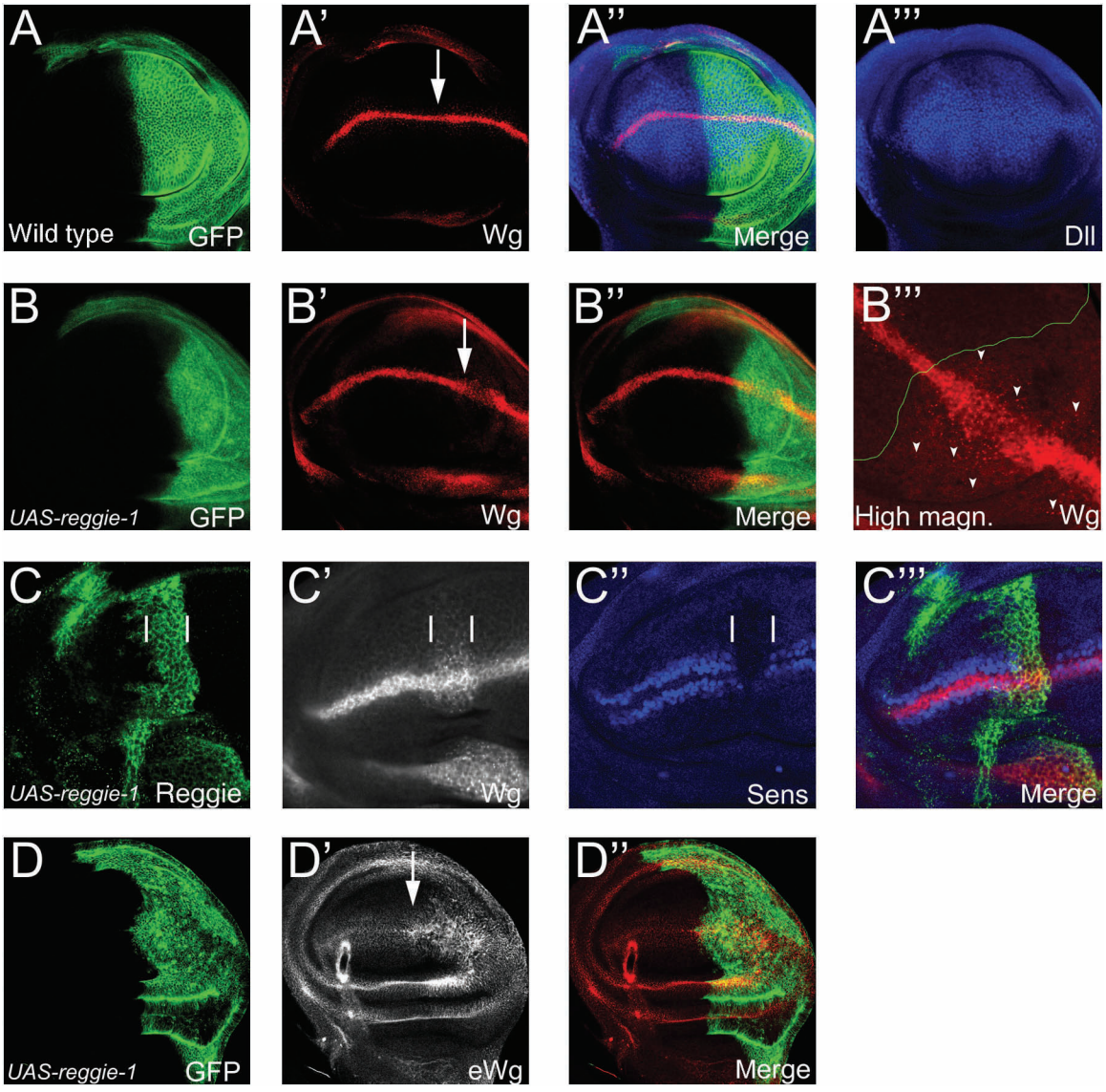

Figure 2 Overexpression of reggie-1 leads to enhanced spreading of Wg. (**A**) Wild-type wing disc expressing GFP in the posterior compartment showing normal Wg and Dll staining. (**B–D**) Overexpression of reggie-1 by *en-Gal4* (B, D) or *dpp-Gal4* (C) results in broadening of the Wg diffusion domain (B′, C′), loss of Sens expression (C″) and vast increase in the extracellular Wg staining (**D′**). White parentheses in panel C mark *dpp-Gal4* expression zone. (B‴) shows a high-power magnification of panel B′; the green line demarcates reggie-1 overexpression. Arrowheads mark Wg puncta appearing far from the production zone.

expression of Homothorax was not induced by reggie-1 (Supplementary Figure S2). Furthermore, expression of a long-range Wg target Distalless (Dll) was not reduced by reggie-1 overexpression (Figure 1H), and in fact was often enhanced (Supplementary Figure S3; Figure 2A‴ shows wild-type Dll expression). Thus, only short-range Wg targets were downregulated by reggie-1. And among those, Sens in the reggie-1-overexpressing domain could be rescued in the vicinity of the wild-type region (Supplementary Figure S4). These observations suggested that reggie-1 was not affecting Wg signal transduction *per se*, but Wg gradient formation.

Overexpression of reggie-1 expands the Wg diffusion gradient

In wild-type discs, the majority of anti-Wg staining is localized close to the stripe of Wg-producing cells, and the concentration of Wg rapidly decays away from the source of production (Figure 2A). Overexpression of reggie-1 by various drivers resulted in erosion of the Wg gradient (Figure 2B and C): Wg could be seen spreading far into the disc at the expense of reduced levels at the source of Wg production. High magnification revealed far-spreading punctate anti-Wg staining (Figure 2B‴); the number of Wg puncta and their apparent size were also increased upon reggie-1 overexpression.

We also found a dramatic autonomous increase in extracellular Wg in the reggie-1 overexpression domain (Figure 2D; Supplementary Figure S5). Wg puncta were not seen without cell permeabilization and thus likely reflected Wg endocytosed by the receiving cells.

Thus, reggie-1 activated Wg secretion and spreading. Erosion of the Wg gradient resulted in reduction of the

short-range targets requiring high levels of Wg, whereas long-range targets were normal or even upregulated.

Reggie-1 changes Wg properties in the Wg-producing and not -responding cells

To analyse whether reggie-1 acted in the Wg-producing or -receiving cells, we restricted reggie-1 overexpression to the domain of Wg production by two independent means. First, we used the *wg-Gal4* line to overexpress reggie-1 only in the endogenous Wg-producing stripe. Remarkably, this reproduced many phenotypes of reggie-1 overexpression in a broader domain: notched adult wings, abnormal Sens expression and reduced wing pouch size (Figure 3G, J and K). Most importantly, Wg gradient also became abnormal: a more diffuse pattern of Wg was seen both looking at total and extracellular anti-Wg staining (Figure 3H and L). Interestingly, the total anti-Wg signal was eroded at the apical level (Figure 3C and I), but unaffected basolaterally (Supplementary Figure S6), indicating that reggie-1 stimulated the apical secretion of Wg, which is the normal way of Wg secretion in polarized cells (Simmonds *et al*, 2001; Pfeiffer *et al*, 2002).

Second, we expressed Wg in somatic clones using the *tub-Gal4* line, either alone or together with reggie-1; in this way we could also exclude the possibility that reggie-1 was indirectly affecting Wg production. Expression of Wg alone in this manner resulted in ectopic activation of the short-range target Sens (Figure 3O). Further, it resulted in downregulation of the endogenous Wg production at the dorso-ventral border (Figure 3N), a phenomenon known to arise from strong overactivation of Wg signalling (Rulifson *et al*, 1996). Remarkably, coexpression of reggie-1 with Wg drastically reduced the capacity of this ectopic Wg to induce ectopic Sens or downregulate endogenous Wg (Figure 3S and T). In contrast, the long-range target Dll was upregulated in both conditions (Supplementary Figure S7). We also

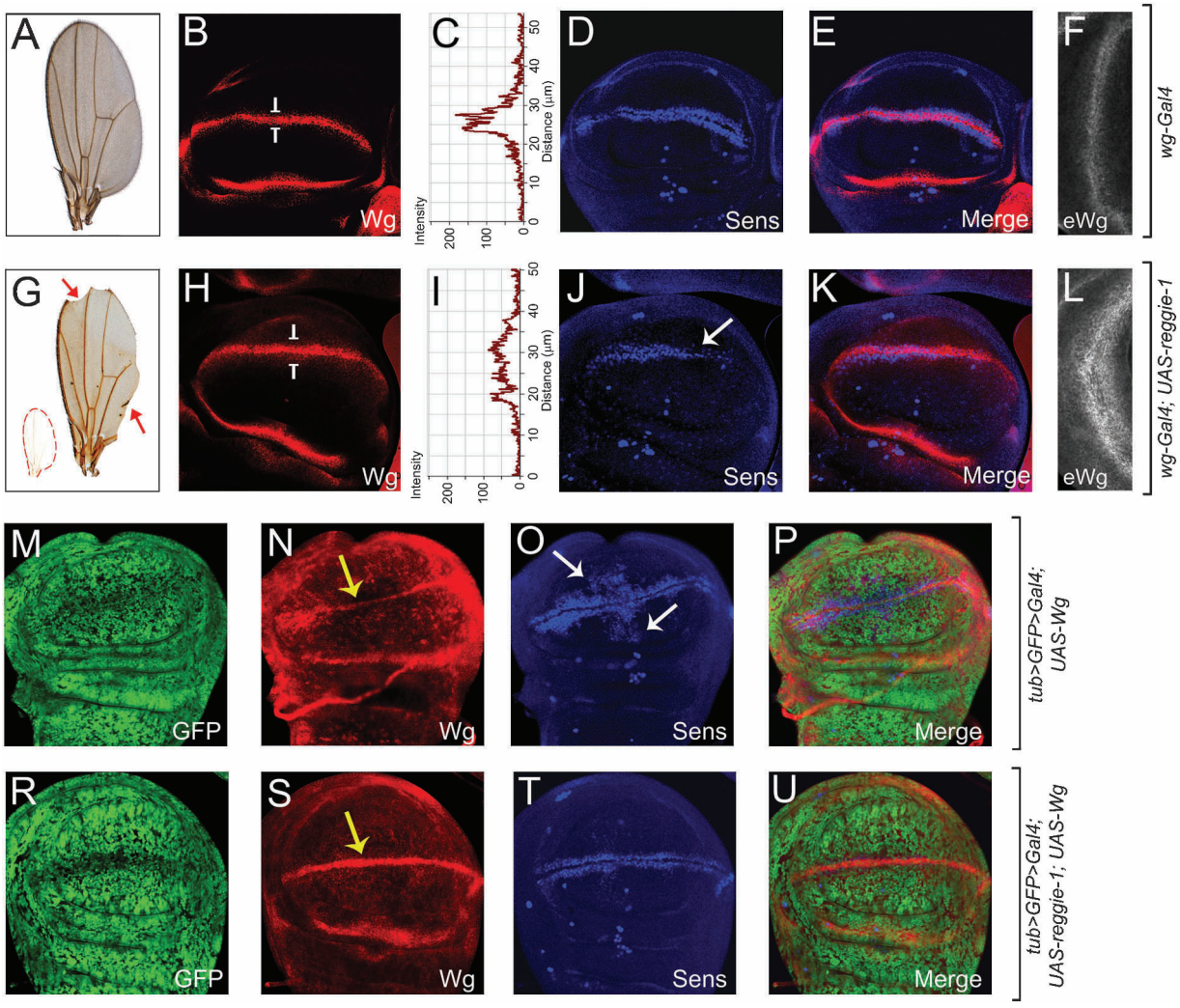

Figure 3 Overexpression of reggie-1 affects Wg-producing cells. (**A–F**) Wild-type (*wg-Gal4*) adult wing (A) and wing disc (B, D–F). (**G–L**) *wg-Gal4; UAS-reggie-1* adult wing (G) and wing disc (H, J–L). Reggie-1 overexpressed by *wg-Gal4* loses wing margin structures (red arrows in panel G), reduces Sens expression and wing pouch size (J, K) and broadens the Wg gradient (H) as compared with wild-type discs (B). Wg gradient is eroded at the apical level: panels C and I show representative pixel intensity scans of wild-type and *UAS-reggie-1* discs; the dorso-ventral border is at position 25 μm. Panels F and L show extracellular anti-Wg stainings oriented at 90° in relation with other panels. (**M–P**) Multiple small *tub-Gal4; UAS-Wg* clones (marked by loss of GFP) induce a reduction in the endogenous Wg production (yellow arrow in panel N) and ectopic Sens induction (white arrows in panel O). (**R–U**) Multiple small clones coexpressing Wg and reggie-1 produce more diffusive Wg (compare the diffuse anti-Wg staining in panel S with localized staining in panel N), incapable to reduce endogenous Wg (S) or induce ectopic Sens (T).

observed enhanced Wg diffusion from the clones coexpressing Wg and reggie-1 (Figure 3).

Thus, overexpression of reggie-1 changes the way Wg-producing cells release Wg. This change apparently involves higher secretion and produces a more mobile form of Wg, affecting gradient formation.

Loss of reggie-1 reduces Wg spreading

Reggie-1 loss-of-function flies are viable and fertile and do not show obvious morphological defects (Hoehne *et al*, 2005). This might be due to redundancy with other reggie-like proteins (Langhorst *et al*, 2005) or compensatory pathways. However, more acute loss of reggie-1 function by expression of a *UAS-RNAi* construct against reggie-1 by *apterous-Gal4* (*ap-Gal4*) or *en-Gal4* resulted in aberrant wings (Supplementary Figure S8).

As reggie-1 overexpression broadened the Wg gradient, we expected the reciprocal phenotypes upon reggie-1 loss of function. Indeed, we found that the gradient of Wg protein, both total and extracellular, was shortened in *reggie-1*$^{-/-}$ wing discs (Figure 4A–D), but that *wg* transcription was unchanged (Figure 4E and F). We found a similar reduction in the Wg gradient when *reggie-1*$^{-/-}$ somatic clones were induced and intersected the Wg production stripe (Figure 4L). High magnification revealed a strong decrease in the amount of Wg puncta emanating from the Wg-producing cells of the *reggie-1*$^{-/-}$ clones (Figure 4L‴). Similar decrease in Wg diffusion and puncta formation could be seen by reggie-1-RNAi expression by *en-Gal4* (Figure 4M and N) and *ap-Gal4* (Supplementary Figure S9). Counting Wg puncta in the RNAi-expressing and wild-type halves of same discs revealed a twofold decrease in Wg puncta formation upon reggie-1 loss of function; the apparent size of Wg puncta was also reduced (Figure 4N and O).

We next analysed expression of Wg target genes in *reggie-1*$^{-/-}$ discs. The domain of the long-range target Dll was narrowed approximately twofold and its intensity reduced in discs deficient for reggie-1 (Figure 4I and J; Supplementary Figure S10); quantification of eight *reggie-1*$^{-/-}$ and wild-type discs stained together revealed a threefold decrease in the Dll expression levels in *reggie-1*$^{-/-}$ (Figure 4K). A decrease in Dll expression was also seen when *reggie-1*$^{-/-}$ clones were induced; this decrease could be non-autonomous resulting from reduced Wg spreading from the *reggie-1*$^{-/-}$ tissue within the Wg-producing stripe (Figure 4L). Narrowing of the Dll expression domain was also seen in the region of reggie-1-RNAi expression (Supplementary Figure S9).

In contrast, *reggie-1*$^{-/-}$ discs had normal expression of the short-range target Sens (Figure 4G and H; Supplementary Figure S10); reggie-1 RNAi also did not prevent Sens expression (Supplementary Figure S9). As a result, wing margin structures were normal in *reggie-1*$^{-/-}$ wings and wings expressing reggie-1-RNAi (see Supplementary Figure S8). Interestingly, in discs homozygous mutant for reggie-1 and heterozygous for a mutation in the *wg* gene, a slight but significant narrowing of the zone of Sens expression could be seen (Supplementary Figure S11).

Thus, reggie-1 is required for the proper Wg gradient formation. The range of Wg spreading and the number of Wg puncta is significantly decreased upon loss of reggie-1, resulting in a strong decrease in expression of the long-range Wg target. In contrast, short-range targets are not affected by loss of reggie-1, unless a concomitant reduction in Wg levels is performed.

Reggie-1 increases Wg and Hh secretion in tissue culture and markedly stimulates Wg endocytosis by co-cultured cells

We next switched to a cell culture to address molecular mechanisms of reggie-1 action. In *Drosophila* cell lines co-overexpressing reggie-1 and Wg, reggie-1 localized to the plasma membrane as well as to intracellular compartments; the Wg signal was mostly intracellular (Supplementary Figure S12). We could observe a limited colocalization of Wg and reggie-1, both intracellularly and at the plasma membrane (Supplementary Figure S12). This limited co-localization might reflect a specific portion of Wg secreted through a reggie-1-dependent pathway. However, by Western blots we did not see a significant increase in Wg secretion upon transfection of Wg-producing S2 cells with reggie-1 (Figure 5A, lanes 5 and 6). Wg secreted by cultured cells can stay attached to the cell membranes and is released by heparan sulphate treatment (Reichsman *et al*, 1996). Heparan extraction produced similar amounts of Wg from control and reggie-1-transfected cells (Figure 5A, lanes 3 and 4). Wg-signalling activity was also not changed by reggie-1 (Supplementary Figure S13).

Western blot analysis is insufficiently sensitive to see small differences. Thus, we used a Wg-*Renilla* luciferase (Rluc) fusion protein to quantify secretion more accurately. We also prepared an Hh-Rluc fusion to monitor whether secretion of Hh might be affected by reggie-1. When we examined the ratio of Rluc activity in media to that in the cells, we observed a statistically significant enhancement of the morphogen secretion by S2R+ cells upon reggie-1 transfection (Figure 5B): Wg levels in the medium increased by approximately 25% and levels of Hh by approximately 50%. However, no significant effect on morphogen secretion was seen by treating the cells with dsRNA against reggie-1 (89 ± 7 and 103 ± 9% of control for Wg and Hh, respectively), indicating that amounts of reggie-1 in S2R+ cells were too low or unimportant for the net morphogen secretion. In contrast, the dominant-negative reggie-1 construct (see Materials and methods) significantly decreased Wg secretion by approximately 20%, potentially affecting other proteins of the reggie-like family (Langhorst *et al*, 2005), but did not have a significant effect on Hh secretion (Figure 5B).

In contrast to Wg and Hh, release of a secreted form of luciferase was not affected by reggie-1 transfection (Figure 5B), demonstrating that reggie-1 did not non-specifically stimulate exocytosis.

We wondered whether the effects of reggie-1 on morphogen secretion could be masked by re-uptake by the cultured cells. Indeed, immunostaining Wg-secreting cells revealed higher intracellular Wg levels upon reggie-1 transfection (not shown). This could be explained by enhanced retention/re-uptake of Wg by the reggie-1-transfected cells. Alternatively, reggie-1 overexpression in the Wg-producing cell could change the properties of this Wg, such that it became more uptakable by surrounding cells regardless of their own reggie-1 levels.

To discriminate between these possibilities, we separately transfected S2 cells with Wg plus reggie-1 (or Wg plus control) and DsRed or EGFP (for independent labelling) and

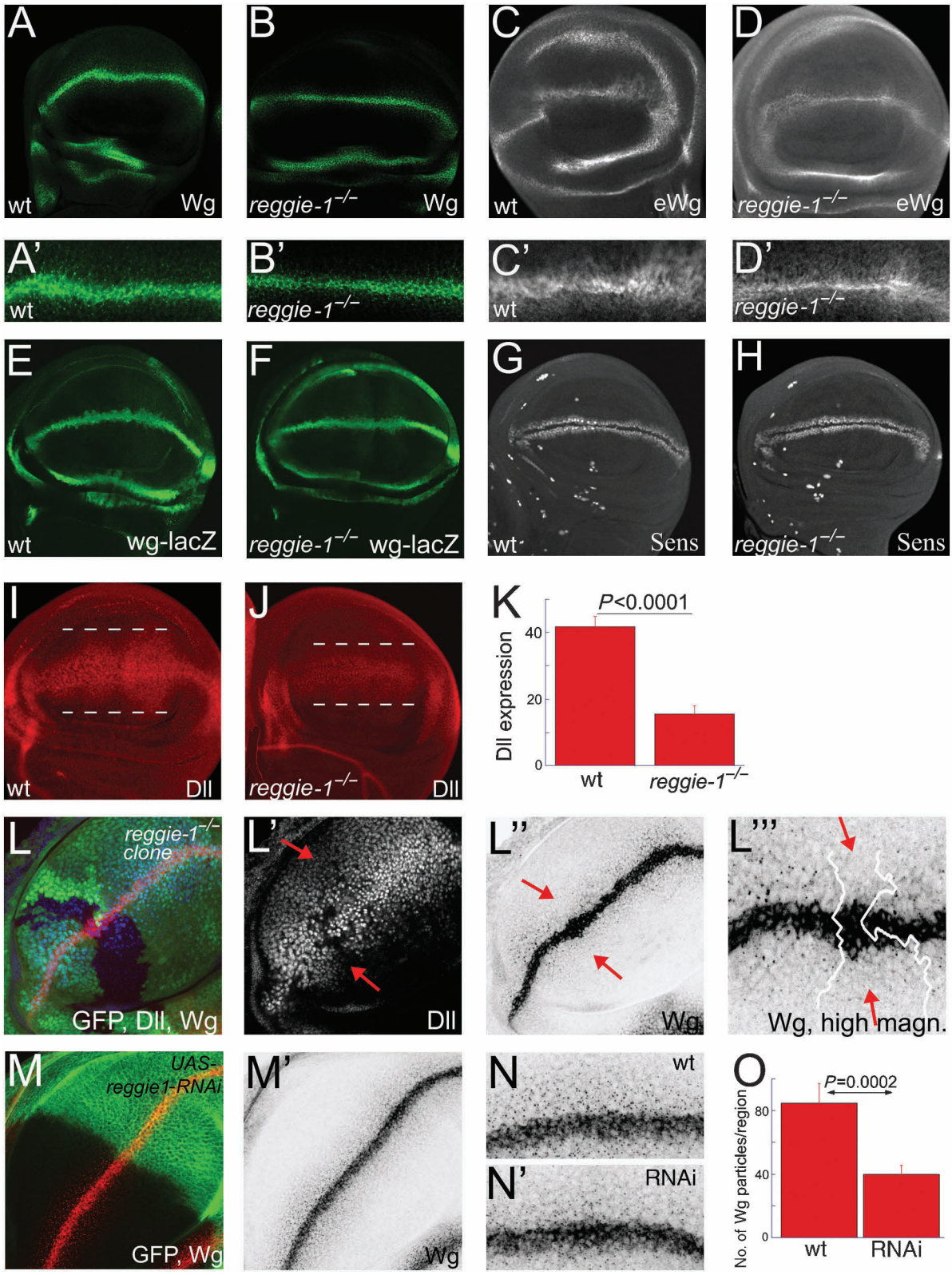

Figure 4 Loss of reggie-1 results in shortening of the Wg gradient and target gene expression. *reggie-1*-mutant discs show more narrow total (**B**) and extracellular (**D**) Wg gradient formation than wild-type discs (**A**, **C**) stained in parallel, but *wg* transcription (**E**, **F**) and Sens expression (**G**, **H**) are unchanged. In contrast, *reggie-1*$^{-/-}$ discs have narrowed Dll expression domains (marked by dotted lines in **I**, **J**) and an overall threefold reduction in Dll expression levels (**K**, data shown as mean$\pm$s.e.m., $n=8$ discs); Student's *t*-test was used to determine statistical significance. (**L**) *reggie-1*$^{-/-}$ somatic clones (marked by loss of GFP) intersecting the Wg-producing stripe reduce Wg spreading (**L**″) and Dll expression (**L**′); the number of Wg particles emanating from the *reggie-1*$^{-/-}$ Wg-producing cells (demarcated by white lines) is reduced (**L**‴). (**M**–**O**) Wg spreading and the number and size of Wg particles are reduced in the domain expressing reggie-1 RNAi (marked by GFP); (N, N′) high-magnification of the wild-type and RNAi-expressing halves of same disc. Wg particles outside the Wg-producing stripe were counted separately in the wild-type and RNAi-expressing halves of identical size of the wing pouch (O); data are shown as mean$\pm$s.e.m., $n=10$ discs; paired *t*-test was used to determine statistical significance.

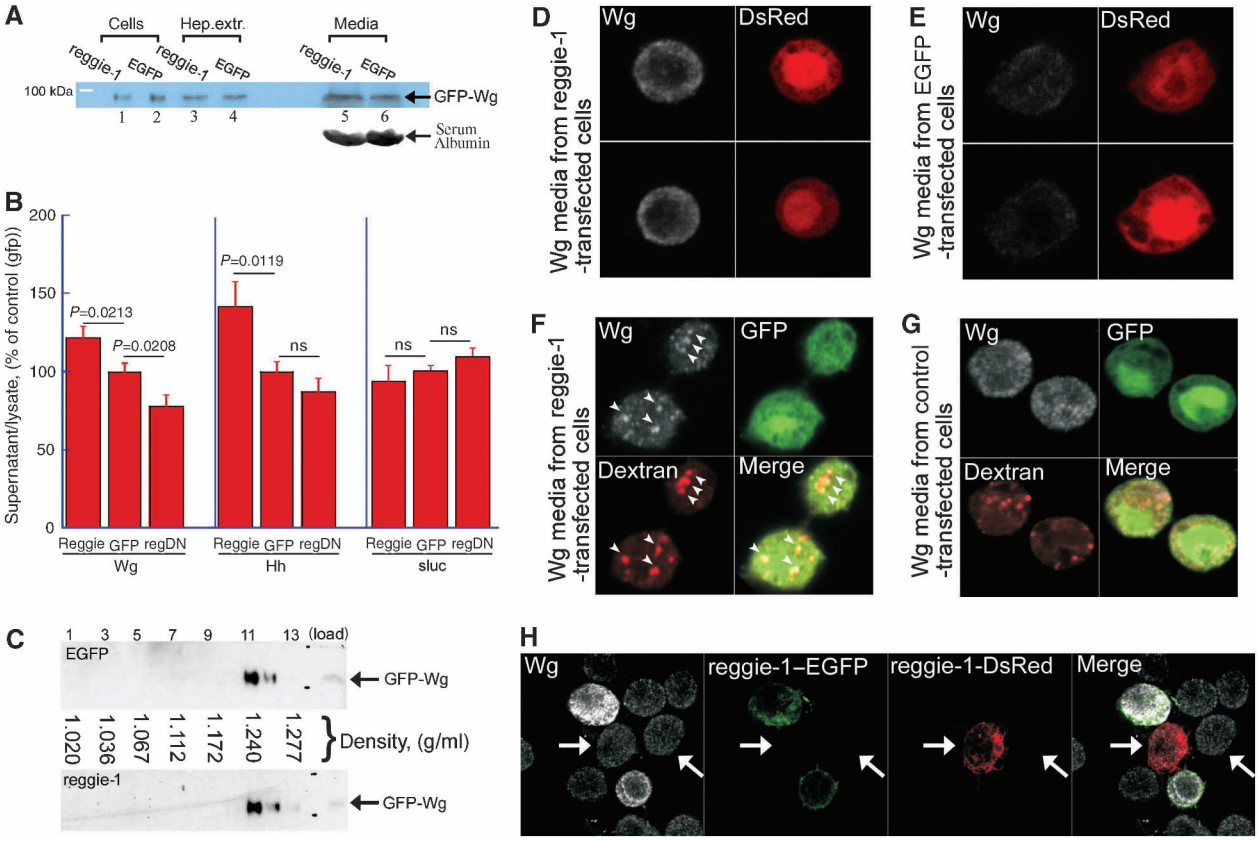

Figure 5 Cell culture experiments analysing the effects of reggie-1 on Wg and Hh secretion. (**A**) Effects of overexpression of reggie-1 on Wg secretion measured by Western blots. Serum albumin is shown as the loading control for the medium samples. (**B**) Effects of overexpression of reggie-1 or a reggie dominant-negative construct on secretion of Wg, Hh or secreted luciferase (sluc) measured by enzymatic assay. Bars represent means ± s.e.m. from 12 experiments. *P*-values from Student's *t*-test are shown; 'ns' means non-significant ($P > 0.05$). (**C**) GFP-Wg media from panel A were subjected to sucrose-density ultracentrifugation. Thirteen fractions were collected (numbers and sucrose density in alternating fractions are shown on top). (**D**, **E**) S2 cells transfected with Wg together with reggie-1 (D) or EGFP (E) were co-cultured with cells independently transfected with DsRed. Accumulation of the anti-Wg signal in DsRed-cells is much higher if Wg was produced by reggie-1-transfected cells. (**F**, **G**) Pulse–chase Texas-red dextran colocalization assays. S2 cells transfected with Wg together with reggie-1 (F) or empty vector (G) were co-cultured with cells independently transfected with EGFP. Accumulation of Wg in dextran-positive early endosomes is much higher if Wg was produced by reggie-1-transfected cells (arrowheads in panel F). Note that unlike in panels D and E where identical confocal settings were used to record anti-Wg staining, the settings in panels F and G were independently optimized for highest resolution. (**H**) Reggie-1 does not stimulate Wg endocytosis cell-autonomously: S2 cells were transfected with Wg plus reggie-1-EGFP and co-cultured with cells independently transfected with reggie-1-DsRed. Incorporation of Wg into the Wg-receiving cells was the same whether they overexpressed reggie-1-DsRed or not (white arrows).

co-cultured these lines. We then analysed whether the co-cultured cells could uptake Wg differently depending on its source. We found a dramatically higher uptake of Wg by DsRed cells when Wg was provided by the reggie-1-over-expressing cells (Figure 5D and E). To prove that this intracellular Wg staining resulted from endocytosis, we performed pulse–chase assays with fluorescent dextran beads and found that Wg provided by reggie-1-expressing cells colocalized with dextran in early endocytic compartments (Figure 5F) whose particulate appearance was reminiscent of the punctate staining seen in wing discs. In contrast, Wg obtained from control-transfected cells was poorly endo-cytosed by the co-cultured cells (Figure 5G). We could also demonstrate that reggie-1 overexpression did not increase Wg uptake cell-autonomously: overexpression of reggie-1 in the cells co-cultured with Wg-producing cells did not increase Wg uptake by these co-cultured cells (Figure 5H). Thus, reggie-1 overexpression was changing the properties of Wg in the Wg-producing cells, such that this Wg became markedly more internalizable by the co-cultured cells. This conclusion agrees well with our *in vivo* observations.

Such increased uptake of Wg might be solely due to enhanced secretion of Wg by reggie-1-overexpressing cells, or also due to a molecular change in the form Wg is secreted. For example, reggie-1 overexpression could enhance Wg packing into lipoprotein particles. To investigate this possibility, we performed sucrose-density ultracentrifugation of the Wg medium from reggie-1- or control-transfected cells (Figure 5C). We found that Wg had identical migration in sucrose gradients in both samples and peaked at the density of approximately 1.24 g/ml; identical results were obtained using GFP-Wg and non-tagged Wg. No signal was detected at the level of lipoprotein particles, which migrate close to the top of the gradient (Panakova *et al*, 2005), despite the fact that the cells were cultured in the presence of bovine serum which contains lipoprotein particles of various sorts (Chapman, 1986). However, it is possible that *Drosophila* Wg can principally not pack into serum lipoprotein particles.

The high density of the Wg signal probably reflects Wg multimers; it is heavier than exosomes, which have the density of approximately 1.13 g/ml (Fevrier and Raposo, 2004).

Thus, in the cell culture we find an effect of reggie-1 on Wg and Hh secretion. Wg produced by reggie-1-transfected cells is more prone to uptake by surrounding cells; the resulting Wg accumulation in early endosomes is similar to the punctate Wg localization stimulated by reggie-1 *in vivo*. The nature of Wg produced by cultured cells is probably multimers, reminiscent of the form mammalian Hh is produced (Zeng *et al*, 2001).

Hh diffusion and target gene expression is affected by reggie-1, whereas Dpp and GFP-GPI are not

The cell culture experiments demonstrate that reggie-1 affects secretion of Wg and Hh, but not luciferase, whereas *in vivo* reggie-1 controls Wg secretion and spreading. We next investigated whether reggie-1 had a similar effect on the spreading of Hh, Dpp and GPI-linked GFP (Greco *et al*, 2001) in wing discs. Whereas Hh was affected by reggie-1 (see below), spreading properties of the Dpp morphogen or GFP-GPI were not changed upon reggie-1 overexpression (Supplementary Figures S14 and S15), arguing that reggie-1 was specifically acting on the Wg and Hh morphogens.

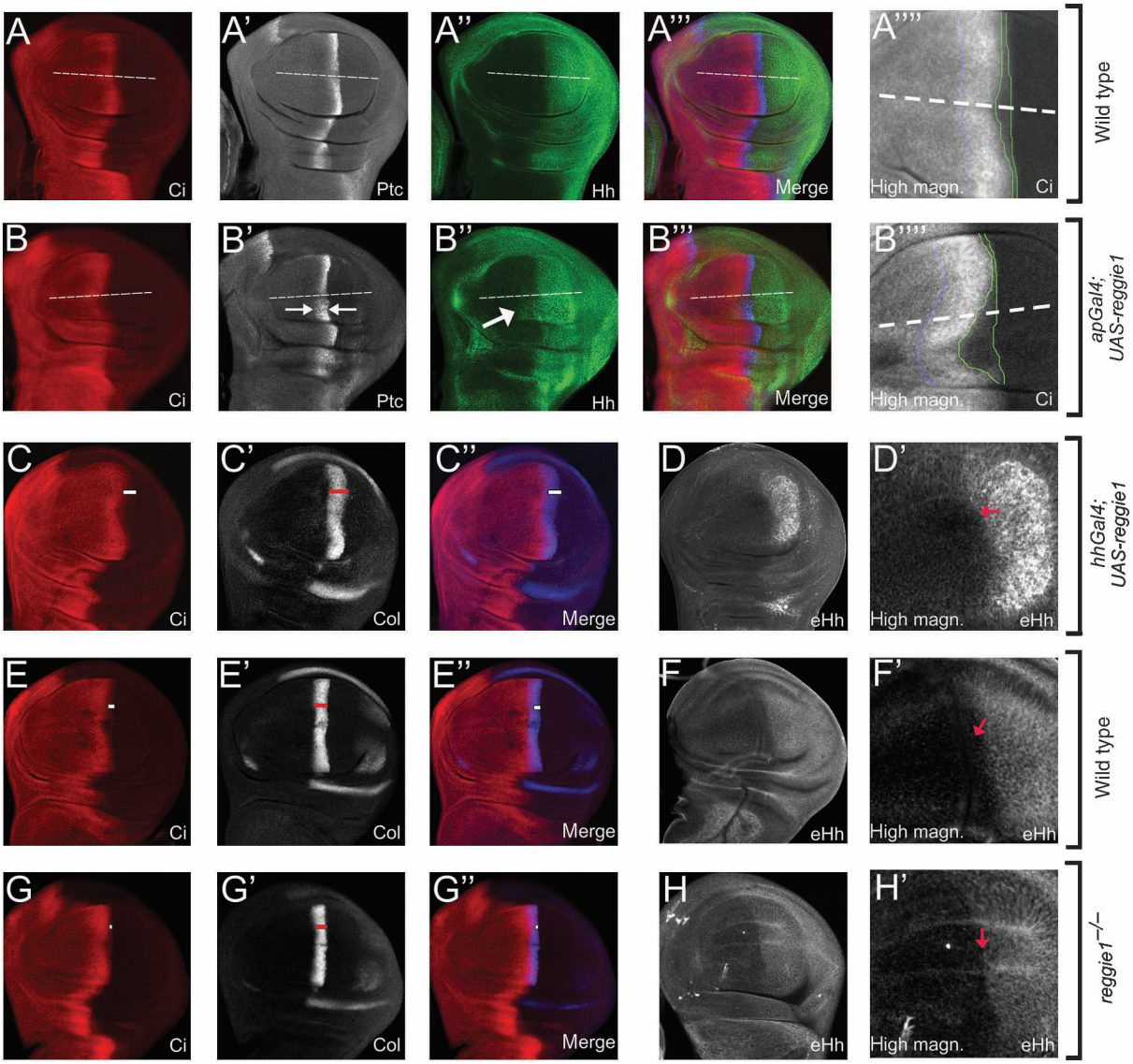

Figure 6 Modulations in reggie-1 levels affect Hh secretion, gradient formation and target gene expression. (**A**, **B**) Wild-type (A) and *ap-Gal4; UAS-reggie-1* (B) discs stained for Ci, Ptc and Hh; *ap-Gal4* drives expression below the white dotted line. Overexpression of reggie-1 results in a dramatic enhancement of Hh spreading into the anterior domain (white arrow (**B**″)) and broadening of the Ptc expression (white arrows (**B**′)). High magnification (**A**‴′, **B**‴′) shows four anti-Ci staining zones, from right to left: (1) no staining in the posterior compartment, (2) low staining representing Ci* (between the two green lines), (3) high staining of Ci-155 (between the green and blue lines) and (4) lower staining in the rest of the anterior representing Ci-75 (left from the blue line). Overexpression of reggie-1 strongly broadens the Ci* zone (B‴′). (**C**–**H**) Wild-type (E, F), *hh-Gal4; UAS-reggie-1* (C, D) and *reggie-1*$^{-/-}$ (G, H) discs stained for Ci (C, E, G), Col (C′, E′, G′), and extracellular Hh (D, F, H). (D′, F′, **H**′) are higher magnifications of panels D, F and H; the red arrows show the range of Hh diffusion into the anterior compartment (beginning of the arrows demarcates the A/P border). (C, C″, E, E″, G, G″) White bars mark the Ci* staining; red bars in (C′, E′, G′) mark Col expression.

Hh is produced by the posterior wing cells and infiltrates the anterior compartment. Cubitus interruptus (Ci) is a crucial transducer of Hh signalling and exists in multiple forms. Depending on the concentration of Hh, Ci*, Ci-155 and Ci-75 forms (listed in the order of high-to-low Hh signalling) can be identified (Methot and Basler, 1999; Figure 6A′′′′). Short-range (like *patched*, Ptc) and long-range (like *dpp*) Hh target genes exist, as well as intermediate targets like *collier* (Col) whose expression overlaps with Ptc and *dpp* (Vervoort *et al*, 1999). We used staining for Hh target genes and the signalling intermediate Ci to monitor Hh signalling levels in response to reggie-1 overexpression or loss of function.

We first overexpressed reggie-1 with *ap-Gal4*, which allowed us to compare the reggie-1-overexpressing dorsal half with the wild-type ventral half within same discs. We found dramatic Hh phenotypes: massive infiltration of Hh into the dorsal anterior domain (Figure 6B′′), and as a result strong thickening of the high-Hh response region expressing Ptc and Ci* (Figure 6B′ and B′′′′). The zone of Ci-155 was shifted to the left from the A/P border, but not broadened (compare Figure 6A′′′′ and B′′′′).

To narrow the effects of reggie-1 to the Hh-producing cells, we overexpressed reggie-1 using *hh-Gal4* and found similar effects: the domain of Ci* was expanded, as well as the zone of Col expression (Figure 6C and E). In adult wings, the outcome was the broadening of the region between veins 3 and 4 (shown in Figure 1B for overexpression of reggie-1 by *en-Gal4*, another posterior expression line). Reggie-1 also induced a dramatic upregulation of extracellular Hh (Figure 6F and D) and enhanced spreading of extracellular Hh into the anterior domain (Figure 6D′ and F′). Thus, similar to our findings concerning the Wg gradient formation, we find that overexpression of reggie-1 increases Hh secretion and spreading, changing the pattern of expression of Hh targets.

We next investigated Hh diffusion and signalling in *reggie-1* loss-of-function. There was a slight reduction in the extracellular Hh in *reggie-1*$^{-/-}$ (Figure 6H) as compared with wild-type discs (Figure 6F) stained in parallel. The A/P border, normally showing a diffuse pattern of extracellular Hh reflecting the diffusion of Hh into the anterior domain (Figure 6F and F′), became considerably sharper in *reggie-1*$^{-/-}$ wing discs (Figure 6H and H′). Thus, spreading properties of Hh produced by the *reggie-1*$^{-/-}$ tissue were reduced. Consequently, the zone of highest Hh signalling represented by formation of Ci* was narrowed (Figure 6G and G′′). Col was not significantly affected (Figure 6G′).

Reggie-1 can affect either short-range or long-range morphogen targets depending on the amount of the morphogen

Morphogen gradient formation can be modelled by a diffusion equation

$$C(x) = \frac{v \cdot w}{2a^2 \cdot \sqrt{D \cdot k}} \cdot e^{-x \cdot \sqrt{\frac{k}{D}}} \qquad (1)$$

where $C(x)$ is morphogen concentration at distance x from the production zone, v is effective morphogen secretion rate, w is width of the production zone, a is cell diameter, k is degradation rate and D is effective morphogen diffusion coefficient (Kicheva *et al*, 2007; Lander, 2007). The wild-type Wg gradient in *Drosophila* wing disc can be modelled using the experimentally determined parameters (Kicheva *et al*, 2007); the threshold Wg concentrations for short- and long-range targets can be roughly set based on the known sizes of Sens and Dll expression domains (Figure 7A and B). We can now simulate the effects of reggie-1 on Wg by varying the diffusion coefficient D: a fivefold decrease in D could faithfully model the Wg phenotypes of reggie-1 loss of function, whereas a fivefold increase in D could simulate the Wg phenotypes of reggie-1 overexpression (Figure 7A and B).

Our experiments clearly show an important role of reggie-1 for formation of the Wg and Hh gradients. However, the functional consequences of changes in reggie-1 levels differed between the two morphogens. For example, overexpression of reggie-1 led to reduction of the Wg short-range targets but expanded the Hh short-range targets. We hypothesized that some of these differences were due to different production levels of Wg and Hh: Wg is produced by a narrow stripe of cells along the dorso-ventral boundary, whereas Hh is produced by the whole posterior half of the disc. Indeed, modelling predicts that broadening of the Wg production zone threefold, with a subsequent fivefold increase in the diffusion coefficient D, will result not in a reduction, but broadening of the short-range targets (Figure 7C); a reciprocal narrowing of the Hh production zone with a concomitant increase of the diffusion coefficient predicts a Wg-like behaviour and reduction in the short-range targets.

To test these predictions, we induced Wg production in the broad zone of the *dpp-Gal4* line. This massive misexpression of Wg induced ectopic Sens (Figure 7D) and Dll expression (not shown). We next co-overexpressed reggie-1 and Wg by *dpp-Gal4*. As expected from the earlier experiments, this resulted in broader diffusion of the ectopic Wg (Figure 7E); further, a more punctate Wg staining was seen (Figure 7E′′ and D′′). As predicted, this broadly produced Wg co-overexpressed with reggie-1 was now fully capable of inducing the short-range target Sens, in a domain broader than when induced by Wg alone (Figure 7E′ and D′). Thus, provided that Wg is in excess, its enhanced mobility does not lead to loss of the short-range targets.

In the case of Hh, we performed the reciprocal experiment reducing Hh amounts. Previous overexpression of reggie-1 by *hh-Gal4* was performed at 17°C and diminished the Hh-producing domain (see Figure 6C). At 25°C reggie-1 overexpression was higher and the posterior domain became even smaller (Figure 7F). Hh spread deeper into the anterior compartment of such discs (Figure 7G and H). Importantly, we found that expression of the intermediate target Col was dramatically narrowed in *hh-Gal4; UAS-reggie-1* discs grown at 25°C, while the domain of the long-range target Ci-155 was significantly broadened (Figure 7F′ and F′′). These changes in expression of short-range versus long-range targets are very similar to what we see when endogenous Wg targets are analysed upon overexpression of reggie-1 (see Figure 1).

Thus, the differential activities of reggie-1 on short-range versus long-range targets of Wg and Hh are determined by the properties of these morphogens, such as the broadness of their production zone. Reggie-1 controls spreading efficiency, but not formation of a particular signalling form of the morphogens, which would be able to induce only long-range targets and not short-range targets.

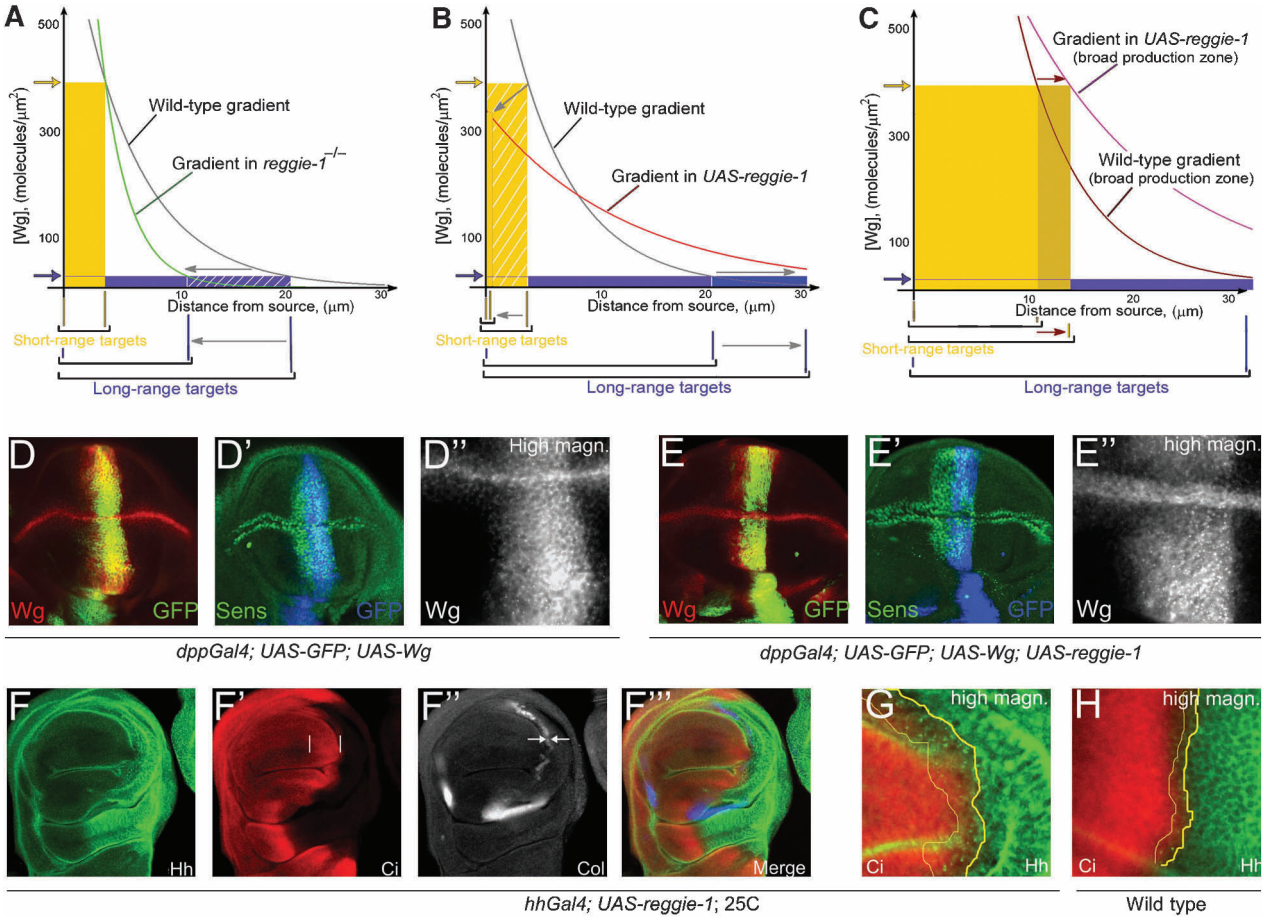

Figure 7 Modulations in reggie-1 levels differently affect short-range and long-range targets of Wg and Hh depending on the morphogen production quantities. (**A–C**) Modelling Wg gradients using Equation 1. The parameters were as follows: $v = 18.7$ molecules/s · cell; $w = 6\,\mu m$; $a = 3\,\mu m$; $D = 0.05\,\mu m^2/s$ and $k = 0.001427\,s^{-1}$ ('wild-type gradient' in panels A and B). D was $0.01\,\mu m^2/s$ in 'gradient in *reggie-1*$^{-/-}$' (A) and $0.25\,\mu m^2/s$ in 'gradient in *UAS-reggie-1*' (**B**, **C**); w was 18 μm in 'broad production zone' gradients (C). Yellow and blue arrows at the y-axis indicate the arbitrarily set [Wg] threshold levels for the expression of the short- and long-range targets respectively. The zone of expression of these targets is indicated by yellow and blue rectangles. Changes in the broadness of the target expression domains in panels A and B are indicated by grey arrows. (**D**, **E**) Ectopic Wg expressed by *dpp-Gal4* (marked by *UAS-GFP*) together with *UAS-reggie-1* spreads further away from the production zone (E), shows a more punctate staining (**E**″) and induces ectopic Sens, and is a broader domain (**E**′) than ectopic Wg expressed without reggie-1 (D–**D**″). GFP staining in (D′, E′) is in blue pseudocolour for a better visualization. (**F**) Expression of reggie-1 by *hh-Gal4* at 25°C strongly reduces the size of posterior compartment and changes Hh responses: Hh now induces narrow Col (arrows in **F**″) but broad Ci-155 (brackets in (**F**′)) zones. (**G**, **H**) High magnification shows enhanced diffusion of Hh into the anterior compartment upon reggie-1 overexpression (G) as compared with wild-type discs (H). The A/P border is shown with a thick yellow line and the range of Hh infiltration is marked with a thin yellow line.

Discussion

With their dual lipid modifications, Wnt and Hh morphogens would be predicted to travel poorly through the extracellular space. Yet they are able to act over long distances, suggesting that specific mechanisms facilitating their spreading must exist, as well as alternative secretion pathways in the morphogen-producing cells, resulting in release of the poorly diffusive and the more mobile pools of the morphogens (Panakova *et al*, 2005; Coudreuse and Korswagen, 2006; Hausmann *et al*, 2007). However, it is unclear how morphogen secretion can be directed for one or the other of the alternative secretion routes.

Here we show that reggie-1/flotillin-2 plays an important role in the morphogen-producing cells, promoting secretion and long-range spreading of Wg and Hh in *Drosophila* wing disc and cell culture. Reggie-1 appears to be specific for these lipid-modified morphogens, as it neither affected spreading of Dpp and GPI-linked GFP in wing discs, nor secretion of an unrelated protein (luciferase) in cultured cells.

The net secretion of Wg and Hh can be stimulated by reggie-1 *in vivo* and in cell culture, whereas changes in the morphogen gradient shapes cannot be explained solely by changes in the morphogen secretion rates. For example, overexpression of reggie-1 erodes the Wg gradient, resulting in a loss of the short-range but not long-range targets, whereas loss of reggie-1 makes the gradient steeper, narrowing the domain of expression of the long-range targets, but not affecting the short-range target genes. From Equation 1 it becomes clear that an increase in the secretion rate can only shift the gradient curve on Figure 7B to the right, to the same extent as broadening of the morphogen production zone achieves (Figure 7C), but cannot erode the gradient. Modelling shows that only an increase in the diffusion coefficient D can erode the gradient such that short-range targets are lost whereas long-range are not, and only a

decrease in *D* can make the gradient steeper such that the long-range targets are narrowed whereas the short-range are unaffected (Figure 7A and B). Thus, reggie-1 does change the way Wg and Hh morphogens are released, promoting their packing for the long-range spreading. The Wg phenotypes we observe in discs can be modelled *in silico* by changing the effective diffusion coefficient *D* fivefold (up for reggie-1 overexpression and down for loss-of-function), giving an idea how significantly reggie-1 changes spreading properties of the morphogens. We predict that reggie-1 stands on one of the two alternative secretion pathways, and thus overexpression of reggie-1 also results in enhanced net secretion. Thus, reggie-1 becomes the first protein identified, which is specifically required within the morphogen-producing cell for secretion of the long-range-spreading forms of Wg and Hh.

Both *in vivo* and *in vitro*, reggie-1 affects the number and intensity of intracellular Wg puncta, which in the cell culture colocalize with dextran beads and can be identified as endosomes. Enhanced endocytosis would be expected to impede morphogen spreading through the tissue, rather than promote it. However, we think that the enhanced endocytosis of Wg we see upon reggie-1 overexpression (or reduced upon reggie-1 loss-of-function), is a secondary effect of the reggie-1 action on morphogen secretion and spreading. Indeed, increased secretion of Wg by the producing cells should already be sufficient to see more Wg endocytosed by the Wg-receiving cells. Moreover, enhanced spreading of the morphogen through the tissue due to increased mobility would allow detect Wg-containing endosomes in Wg-receiving cells further away from the source of production. Thus, the number and brightness of the Wg-containing intracellular puncta is rather a consequence and readout of Wg secretion and spreading under the control of reggie-1.

It is not clear whether reggie-1 promotes packing of Wg and Hh into lipoprotein particles (Panakova *et al*, 2005) or high-order multimers (Zeng *et al*, 2001), or into another yet unidentified form promoting efficient spreading. Our experiments *in vitro* suggest that Wg is multimerized by cultured cells. In wing discs, loss of reggie-1 produces stronger phenotypes on Wg long-range target activation than the previously reported loss of lipoprotein particles: a threefold reduction in Dll expression is observed in *reggie-1*$^{-/-}$ discs (Figure 4K), compared with a slight decrease in the lipoprotein-RNAi animals (Panakova *et al*, 2005). However, additional experiments are required to determine whether the S2 cells-derived Wg could associate with lipoprotein particles of *Drosophila* (rather than serum) origin, and whether *in vivo* effects of reggie-1 are lipoprotein-dependent or not. It is also possible that the reggie-dependent pathway is redundant with another way of long-range morphogen spreading, and removal of both might produce additive effects.

It is also possible that the reggie-dependent pathway may utilize additional reggie-related proteins. Reggie-2 is unstable in the absence of reggie-1 (Hoehne *et al*, 2005; Solis *et al*, 2007) and reggie-2 overexpression or loss-of-function produces no phenotypes ((Hoehne *et al*, 2005) and this work). However, a certain redundancy with other members of the SPFH (Stomatin/Prohibitin/Flotilin/HflK/C) protein family (Tavernarakis *et al*, 1999) might be expected (Langhorst *et al*, 2005). Eleven *Drosophila* genes, including reggie-1 and -2, are listed in InterPro as encoding proteins with the SPFH domain (also known as Band 7 domain); most of these genes are poorly studied and described in the Flybase as components of the cytoskeleton based on electronic annotation.

Reggie-1 and possibly other members of the SPFH family function as protein platforms for organizing specific types of membrane microdomains (lipid rafts) (Langhorst *et al*, 2005; Stuermer and Plattner, 2005). Based on our observations, we can speculate that association of Wg and Hh with reggie-1-based membrane microdomains is crucial for the efficient formation of the mobile forms of these morphogens. We could observe partial colocalization of Wg and reggie-1 in cultured cells, but the exact identification of the intracellular compartment(s), where reggie-1-dependent secretion of Wg and Hh occurs, requires additional investigation.

In conclusion, we have uncovered an important function of the membrane microdomain-scaffolding protein reggie-1 in secretion and gradient formation of the lipid-modified Wg and Hh morphogens in *Drosophila*. Reggie-1 is the first protein identified, which is specifically required *in cis* for the long-range-directed spreading of these morphogens. This illustrates that in the morphogen-producing cell, multiple secretion pathways and ways of morphogen packing exist, allowing proper activation of the short- and long-range target genes.

Materials and methods

Genetics

The *Drosophila* lines used are as follows: *UAS-reggie-1*, *UAS-reggie-2* and *reggie-1*$^{-/-}$ (*Flo-2*^{KG00210}) (Hoehne *et al*, 2005); *en-Gal4, UAS-GFP-GPI* (Greco *et al*, 2001); *hh-Gal4* (Tanimoto *et al*, 2000); *wg-Gal4* (ND382) (Gerlitz *et al*, 2002); *tub>y+ GFP>Gal4* (gift from G Struhl); *UAS-Arm*Δ*RGS* (Willert *et al*, 1999); *UAS-cyclinE* (Johnston and Edgar, 1998); *UAS-GFP-Dpp* (Entchev *et al*, 2000); *UAS-RNAi-reggie-1* and *UAS-RNAi-reggie-2* (Dietzl *et al*, 2007). *MS1096-Gal4*; *ap-Gal4*; *dpp-Gal4*; *wg-lacZ* (*wg*^{en11}) were from Bloomington Stock Center. Mitotic clones were induced by the Flp-mediated recombination (Golic and Lindquist, 1989) between *reggie-1*$^{-}$, *frt19A* and *ubi-GFP, frt19A* chromosomes using *hsp70-flp* (Struhl and Basler, 1993). Heat-shock (37°C) was applied for 1 h, 12–36 h after egg laying for *reggie-1*$^{-/-}$ clones and for 30 min, 48–72 h after egg laying for *tub>Gal4* clones. Flies were kept at 25°C; crosses for Figure 6 were performed at 17°C.

Histology

Wing discs from late third instar larvae were fixed in 3.7% formaline, permeabilized in 0.5% NP-40 and immunostained in 0.2% Tween 20 in PBS, followed by confocal microscopy. For extracellular staining, permabilization was omitted; discs were incubated 30 min on ice with a triple antibody concentration before fixation. BrdU staining was performed as described in Johnston and Edgar (1998).

Antibodies used are as follows: guinea pig anti-Sens 1:1000 (Nolo *et al*, 2000) and anti-Hth 1:1000 (Abu-Shaar *et al*, 1999); rat anti-BrdU 1:200 (Serotec) and anti-Ci 1:20 (Motzny and Holmgren, 1995); rabbit anti-Dll 1:100 (gift from R Mann), anti-reggie-1 (R722) 1:100 (Hoehne *et al*, 2005) and anti-Hh (NHhI) 1:1000 (Takei *et al*, 2004); mouse anti-β-gal 1:100 (Promega) and anti-Col 1:50 (gift from A Vincent and M Crozatier). Mouse antibodies against Cut (1:30), Wg (1:50) and Ptc (1:10) were from DSHB. FITC-, Cy3- and Cy5-labeled (Jackson ImmunoResearch) and Alexa Fluor® 405 (Invitrogen) secondary antibodies were used.

reggie-1$^{-/-}$ and *en-Gal4, UAS-GFP* larvae were mixed and their discs stained together for precise comparison of wild-type and *reggie-1*$^{-/-}$ phenotypes; the same procedure was followed for *UAS-reggie-1* and wild-type discs; GFP and anti-reggie-1 were used to distinguish the genotypes.

Molecular cloning

The following constructs were cloned into pAc5.1/V5-His (Invitrogen):

- reggie-1–EGFP: RT–PCR product (primers CGAATTCATGGGCAA CATACACACGACGGGTCCC, CACCGGTGCCTTGGCACCCGGTATC TTGGACAGA) from total S2 cell RNA was cloned into pCRII-TOPO and then pEGFP-N1;
- reggie-1 untagged cloned directly from pCRII-TOPO above;
- reggie-1-dominant negative–EGFP: was created analogously to the one in rat (Langhorst *et al*, 2006) and included aa 184–386;
- EGFP and DsRed-monomer cDNAs (BD Biosciences);
- reggie-1-DsRed-monomer fusion;
- Rluc fusion: was generated replacing the GFP-coding sequence of Wg-GFP with the Rluc coding sequence;
- a secreted form of luciferase (sLuc): was generated by fusing the signal peptide of haemagglutinin (MAIIYLILLFTAVRG) to the firefly luciferase coding sequence.

dsRNA targeting the coding sequence reggie-1 was generated using the Ambion Megascript kit and primers containing a T7 polymerase-binding site plus the following sequence-specific primer sequences: ACGCTTACAGTAGAAGAGG, CTTGGCCTGCGCAAGG GTCTGG; CAACGATGTGACTCGCTTGG, GAGTACATTTAGCTACACG TTCG; CAAGAAGCGCACGATTGTGG, GTATCTTGGACAGAACCTT GG. dsRNA treatment was performed as in Worby *et al* (2001) and induced loss of anti-reggie-1 staining in reggie-1-transfected cells.

Cell culture

S2, S2R+, S2-GFP-Wg (Piddini *et al*, 2005) and Kc167 cells were grown at room temperature in Schneider's medium with 10% foetal bovine serum, 1% glutamine and 1% penicillin–streptomycin, and transfected using CellFECTIN (all from Invitrogen).

S2-GFP-Wg cells were transfected with reggie-1–EGFP or EGFP for 24 h, washed 3× PBS and the expression of GFP-Wg was induced by 0.5 mM $CuSO_4$ for 24 h. For heparan extraction, the cells were washed 3× with PBS and treated with 10 μg/ml of each heparan and chondroitin sulphate A (Sigma) in serum-free medium for 2 h. For sucrose-density ultracentrifugation, 200 μl of media were overlaid on top of 150 μl of 25% sucrose overlaid on top of 45% sucrose over 80% sucrose (all in PBS), and centrifugation was performed at 268 000 *g* 48 h 4°C in a swing-out rotor. 50-μl volume fractions were collected and sucrose density was determined by refractometry. Western blot was performed using mouse anti-Wg (DSHB) at 1:500.

For the activity assays 48 h post-transfection, cells in the conditioned medium were mixed with S2 cell line stably transfected with DFz2, LEF and a LEF-luciferase reporter (Schweizer and Varmus, 2003), and cultured for additional 24 h before harvesting and measuring luciferase values using the Dual Luciferase Assay System (Promega).

For Hh secretion assays, the UAS-Hh-Rluc construct was cotransfected with Ac-Gal4 (Ma *et al*, 2002). The control sLuc construct was cotransfected with the Wg- or Hh-Rluc fusions. The levels of Rluc and sLuc in the supernatant and cell lysates at 48 h were measured. The sLuc/Rluc ratios were determined to normalize for transfection efficiency.

For studies of Wg uptake, S2 cells were separately cotransfected with Wg plus reggie-1-EGFP or Wg plus EGFP, and another cell population was transfected with DsRed. Three hours post-transfection, the cells were washed 3× with PBS, co-cultured for 24 h and prepared for immunofluorescence.

For endocytosis assays following Entchev *et al* (2000), S2 cells were separately cotransfected with Wg plus reggie-1 or Wg plus empty vector, and another cell population was transfected with EGFP. Three hours post-transfection, the cells were washed 3× with PBS and co-cultured for 24 h. Then, cells were incubated on ice with 0.5 mM Texas-red dextran (lysine fixable, M_r 3000; Molecular Probes) in the medium for 10 min (pulse) and washed 3× with cold PBS. This was followed by immediate fixation or incubation with fresh medium at 26°C for 20 min (chase) to visualize early endocytic compartments with subsequent fixation.

For colocalization studies, S2R+ cells were cotransfected with Wg and reggie-1-EGFP for 24 h, adhered to Alcian blue-coated coverslips, fixed and immunostained.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

Acknowledgements

We thank the researchers listed in the Materials and methods section for sharing fly strains, antibodies and cell lines. The work was supported by the Deutsche Forschungsgemeinschaft (SFB-TR11) to V.L.K. and C.A.O.S., and Swiss National Science Foundation to K.B.

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