In vivo role of lipid adducts on Wingless

Xavier Franch-Marro[1,*], Franz Wendler[1,*], Janice Griffith[2], Madelon M. Maurice[2] and Jean-Paul Vincent[1,‡]

[1]National Institute for Medical Research, The Ridgeway, Mill Hill, London, NW7 1AA, UK
[2]Department of Cell Biology, University Medical Center Utrecht (UMCU), Rm G02.525, Heidelberglaan 100, 3584CX Utrecht, The Netherlands
*These authors contributed equally to this work
‡Author for correspondence (e-mail: jvincent@nimr.mrc.ac.uk)

Accepted 3 March 2008
Journal of Cell Science 121, 1587-1592 Published by The Company of Biologists 2008
doi:10.1242/jcs.015958

Summary

Two lipids (palmitate and palmitoleic acid) are appended onto Wnt proteins. It has been suggested that palmitate is required for signalling, whereas palmitoleic acid is necessary for progression through the secretory pathway. By mutating the relevant amino acids, we have investigated how these adducts contribute to the secretion and signalling activity of Wingless, the main *Drosophila* member of the Wnt family. Analysis of Wingless with a Cysteine 93 to Alanine mutation ([C93A]Wingless) shows that palmitoylation is essential for signalling activity in vivo (as well as in cultured cells). Moreover, without palmitate, Wingless fails to reach the surface of imaginal disc cells and, as electron microscopy (EM) analysis suggests, appears to accumulate in the endoplasmic reticulum (ER). Artificial targeting of palmitate-deficient Wingless to the plasma membrane does not rescue signalling activity. Therefore, palmitate at C93 has a dual role: in secretion and signalling. From our analysis of [S239A]Wingless, which lacks a conserved residue shown to be acylated in Wnt3a, we infer that palmitoleic acid is not, as previously suggested, absolutely required for secretion. Nevertheless, this mutant has poor signalling activity, suggesting that palmitoleic acid contributes significantly to signalling. We suggest that the overall level of lipidation affects signalling activity.

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/121/10/1587/DC1

Key words: Wingless, *Drosophila*, Secretion, Acylation, Morphogen

Introduction

Lipid modification has recently emerged as an important feature of several signalling molecules. Lipid-modified secreted proteins include the members of the Hedgehog (Hh) and Wnt families, and, as shown recently, the *Drosophila* homologue of TGFα, Spitz (Spi) (Miura and Treisman, 2006). Two distinct lipids are appended on Hh proteins: palmitate at the N-terminus and cholesterol at the C-terminus (Pepinsky et al., 1998); however, so far, Wnts and Spi are only known to be acylated (Willert et al., 2003; Miura et al., 2006; Takada et al., 2006). The hydrophobic nature of secreted lipoproteins raises interesting questions concerning progression through the secretory pathway, release from the surface of producing cells and transport to distant cells within a tissue. In this paper, we investigate how lipid modifications affects the secretion and signalling activity of Wingless, a *Drosophila* member of the Wnt family.

Two lipids have been shown to be appended onto Wnts: a palmitate on a cysteine and a palmitoleic acid on a serine. Palmitoylation at cysteine 93 (C93) of Wingless has been demonstrated by Willert et al. (Willert et al., 2003). The corresponding residue of mouse Wnt3a (C77) is also palmitoylated, suggesting that this modification is conserved. Indeed, replacement of this residue by alanine (C93A in *Drosophila* or C77A in the murine protein) leads to near complete loss of signalling activity in a cell-based assay (Willert et al., 2003). Recently, it has been shown that Wnt1 and Wnt5a are also palmitoylated and that this modification is essential for the signalling activity of this Wnt family member (Galli et al., 2007; Kurayoshi et al., 2007; Miura and Treisman, 2006). Because overexpressed [C77A]Wnt3A has residual activity in an autocrine-cell-based assay, it has been suggested that palmitoylation might not be essential for signalling activity per se but would rather be needed to concentrate the signal at cell membranes. Indeed, in the absence of palmitate at C77, Wnt3a becomes more soluble in aqueous media (Willert et al., 2003).

In a recent study, Takada et al. showed that Wnt3a is acylated at a second residue: Serine 209 (S209) is modified with palmitoleic acid (Takada et al., 2006). Because this residue is conserved, it is likely that the same modification takes place in *Drosophila* (at position 239). Importantly, Takada et al. show that, without this modification, Wnt3a is no longer secreted. Because [C77A]Wnt3a is secreted, at least in a cell-based assay (Willert et al., 2003), Takada et al. suggest that the two lipids on Wnt3a could serve distinct functions, with palmitic acid on C77 being involved in signalling activity and palmitoleic acid at S209 being required for progression through the secretory pathway.

In this study we use wing imaginal discs of *Drosophila* as an in vivo system in which to study the role of the acyl groups at C93 and S239 in Wingless. During the third-instar larval stage, Wingless is normally expressed in a row of cells that bisects the disc along the dorsoventral boundary. From there, it spreads throughout most of the presumptive wing region, where it binds to receptors of the Frizzled family (mainly Frizzled 2; Fz2) and activates downstream signalling. We find that, in imaginal discs, lipid modification at S239 is not absolutely required for secretion or membrane association but contributes to signalling activity. By contrast, mutation of C93 (no palmitoylation) causes inefficient exit from the endoplasmic reticulum (ER) and abrogates signalling. Our results suggest that dual lipidation is required for signalling activity. Lipidation also contributes to trafficking through the secretory pathway, although, in *Drosophila* Wingless, the role of palmitate predominates over that of palmitoleic acid, whereas the contrary is true for mammalian Wnt3a.

Results and Discussion

Acylation is required for signalling activity in vitro and in vivo

As reported by Galli et al. (Galli et al., 2007), Willert et al. (Willert et al., 2003) and Kurayoshi et al. (Kurayoshi et al., 2007), respectively, palmitoylation of Wnt1, Wnt3a and Wnt5a at an N-terminal cysteine is essential for signalling activity. We found that a mutation at the corresponding site in Wingless (C93A) abolishes signalling activity in cultured *Drosophila* S2 cells. S2 cells were co-transfected with a Fz2-expressing plasmid, a reporter plasmid (topflash) and a plasmid encoding either wild-type Wingless or [C93A]Wingless. A plasmid constitutively expressing *Renilla* luciferase was also included for normalisation and to provide a measure of cell health. Normally, three different forms of Wingless, representing different levels of glycosylation, can be distinguished by western blot (Tanaka et al., 2002). We found that [C93A]Wingless is also present in three forms (Fig. 1A), suggesting that it is expressed and modified normally. Nevertheless, C93A did not show any significant signalling activity (Fig. 1B). Using similar assays, we found that [S239A]Wingless is also modified normally by disc cells (Fig. 1A). Surprisingly, considering that [S209A]Wnt3a is inactive (Takada et al., 2006), [S239A]Wingless was found to have residual signalling activity (Fig. 1B).

In order to assess the in vivo significance of these in vitro observations, we generated transgenic flies expressing either Gal4-inducible HA-tagged [C93A]Wingless (*UAS-HA-[C93A]Wingless*) or [S239]Wingless (*UAS-HA-[S239]Wingless*). For control experiments, we used flies expressing HA-tagged wild-type Wingless (*UAS-HA-Wingless*). Strains expressing comparable amounts of protein, as assessed by western blot, were selected (data not shown). The transgenes were expressed under the control of the *ap-Gal4* driver, which is active in the dorsal compartment of wing discs, including in one of the two rows of cells that normally express Wingless (see diagram in Fig. 1C). Using this driver, HA-Wingless causes 100% lethality at the pupal stage (not shown) (see Struhl and Basler, 1993). By contrast, expression of HA-[C93A]Wingless had no detectable effect: resulting flies were viable and no morphological defects could be seen even though mild activation of the Wingless pathway in the prospective wing blade causes the formation of ectopic bristles (Greaves et al., 1999). We conclude that [C93A]Wingless has no signalling activity in vivo. In the same assay, HA-[S239]Wingless prevented complete fusion of the notum but had no effect on wing patterning (e.g. no ectopic bristles). This is consistent with the weak activity seen in S2 cells. We conclude that palmitoylation at C93 is essential for signalling and that acylation at S239, although not essential, contributes significantly to signalling activity.

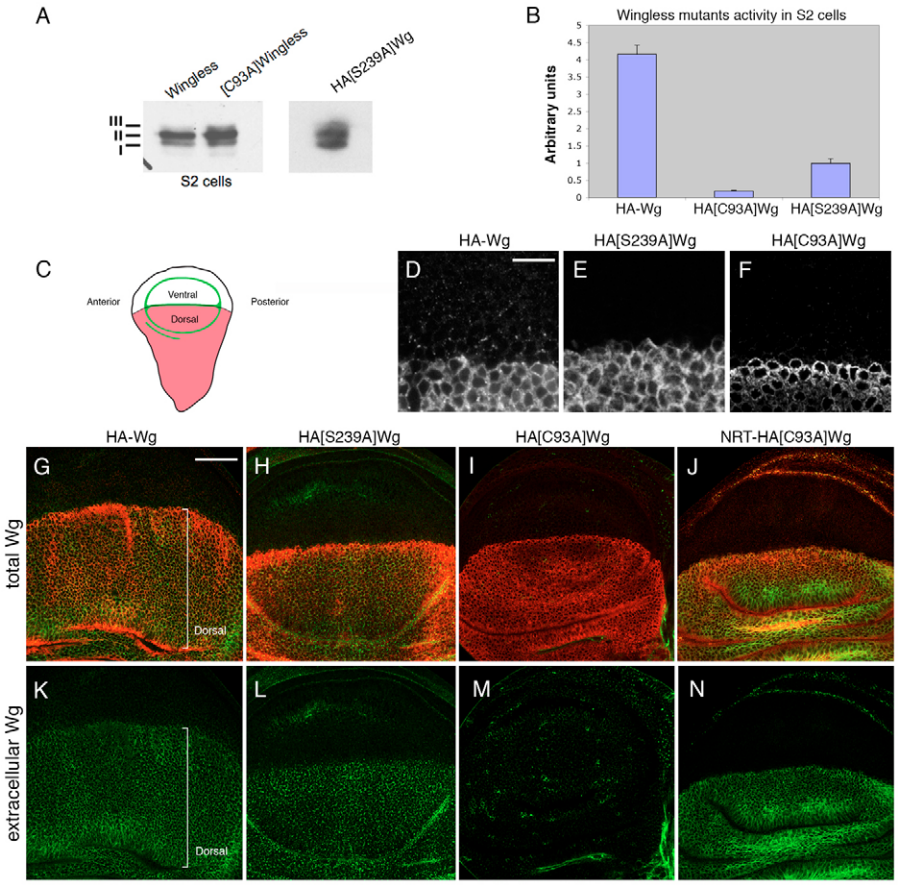

Fig. 1. (A) Expression and post-translational modifications of [S239] and [C93A]Wingless. *Drosophila* S2 cells were transfected with plasmids expressing either the wild-type or the [C93A]Wingless protein and processed for immunoblotting (left lanes). Right-hand-side lane shows [S239]Wingless obtained from transgenic imaginal discs. Distinct bands representing different states of glycosylation can be detected in all three cases. (B) [C93A]Wingless has no significant signalling activity, and that of [S239]Wingless is strongly reduced. *Drosophila* S2 cells were transfected with the topflash reporter along with plasmids expressing Fz2 and *Renilla* luciferase. The cells were also transfected with equal amounts of plasmids expressing [S239]Wingless, [C93A]Wingless or wild-type Wingless. Luciferase activity was measured and normalised against *Renilla* activity for the two groups of cells. (C) Diagram of a wing imaginal disc showing the domain of Wingless expression in green and of ap-Gal4 in red. (D-F) Expression of wild-type Wingless (D), [S239]Wingless (E) and [C93A]Wingless (F) under the control of *ap-Gal4*. Unlike the wild-type protein, the mutant proteins cannot be detected in non-expressing cells (no punctate staining can be detected). Scale bar: 10 μm. (G-N) Extracellular staining versus total staining of various Wingless forms expressed under the control of *ap-Gal4*. In all panels, total staining is shown in red and extracellular staining in green. Scale bar: 50 μm. (G,K) Expression of wild-type Wingless. Note the presence of extracellular signal at the surface of expressing cells, in the dorsal compartment (square brackets). The pouch is enlarged because of excess activation of Wingless signalling. (H,L) Expression of [S239]Wingless. Strong extracellular staining can be detected in the expression domain. (I,M) Expression of [C93A]Wingless. No extracellular signal is detected. (J,N) Expression of NRT-[C93A]Wingless. Extracellular localisation is restored by the presence of a heterologous transmembrane domain.

Distribution of [C93A]Wingless and [S239A]Wingless in imaginal discs

The distribution of wild-type Wingless, [S239A]Wingless or [C93A]Wingless was assessed by immunofluorescence following expression in imaginal discs with *ap-Gal4*. Using a conventional staining protocol that reveals both intra- and extra-cellular proteins, HA-tagged wild-type Wingless was detected in non-expressing cells,

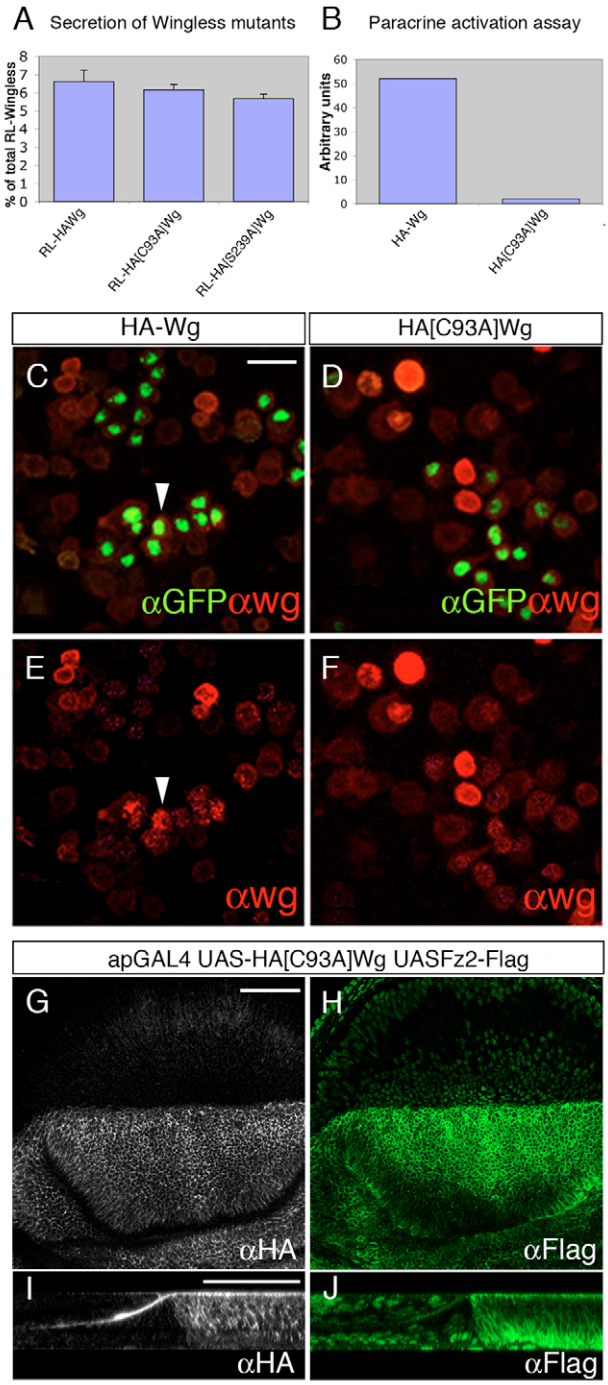

Fig. 2. Secretion of wild-type and mutated Wingless in vitro and in vivo. (A) *Drosophila* S2 cells were transfected to express wild-type, [S239] or [C93A]Wingless fused to *Renilla* luciferase (RL) protein. The graph shows luciferase activity as a percentage of the total activity present in cell extracts and in the supernatant. No significant difference is seen between the three samples. Cells also expressed cytoplasmic firefly luciferase. In all cases, less than 2% activity was detected in the medium, indicating very limited leakage. (B-F) 'Donor' cells were transfected with either wild-type or [C93A]Wingless together with *Renilla* luciferase for normalisation. 'Receiving' cells were transfected with Fz2 and topflash. A histone-GFP plasmid was also included in the transfection mix in order to recognise receiving cells in subsequent immunofluorescence experiments. (B) First, a subset of the cells was processed for the topflash assay and luciferase activity was normalised to *Renilla* activity. As expected, wild-type Wingless causes high reporter activity in this paracrine assay, whereas [C93A]Wingless does not induce detectable activity over background. (C-F) In parallel, both donor and receiving cells were mixed and processed for immunofluorescence. Anti-Wingless is shown in red and receiving cells are green. Wild-type Wingless can be seen in receiving cells both at the cell surface and in intracellular vesicles (arrowhead in C and E). (D,F) By contrast, no HA-[C93A]Wingless can be seen in receiving cells. Scale bar: 10 µm. (G-J) Fz2 overexpression causes mild accumulation of [C93A]Wingless at the cell surface. Fz2 (Flag) and [C93A]Wingless (HA) were co-expressed with *ap-Gal4*, and imaginal discs were stained with the extracellular protocol. [C93A]Wingless is now detectable at the cell surface (G; compare with Fig. 1M, in which Fz2 is not co-expressed). An optical cross-section (along the dorsoventral axis) shows that [C93A]Wingless tends to accumulate in the apical half of the cell (I), whereas Fz2 is present across the apicobasal axis (J). Scale bars: 50 µm.

in which it is known to be internalised (top half of Fig. 1D) (see also Piddini et al., 2005). By contrast, neither [S239A]Wingless nor [C93A]Wingless could be detected outside the expression domain (Fig. 1E,F). Therefore, reduction in lipidation prevents Wingless from reaching target cells. Using a staining protocol designed to reveal only extracellular proteins, we confirmed that wild-type Wingless accumulates at the surface of expressing cells (Strigini and Cohen, 2000) (Fig. 1G,K). [S239A]Wingless also accumulated at the cell surface (Fig. 1H,L); hence, it must be able to progress through the secretory pathway. By contrast, no [C93A]Wingless could be detected at the surface of expressing imaginal disc cells (Fig. 1I,M). This suggests that secretion of this form of Wingless is impaired in vivo. Alternatively, [C93A]Wingless could be secreted but not retained at the cell surface. Overall, our in vivo results differ somewhat from those obtained in mammalian cell culture. In particular, in our assay, palmitate at C93 of Wingless appears to be more important for secretion than palmitoleic acid at S239.

Failure to reach, or accumulate at, the cell surface could explain why [C93A]Wingless is unable to signal. To test this possibility, palmitoylation-deficient Wingless was tethered at the cell surface with a heterologous transmembrane domain from the Neurotactin protein (NRT). We expressed NRT-HA-[C93A]Wingless in transgenic imaginal discs and compared its signalling activity to that of wild-type protein similarly tethered to the cell membrane; the latter is known to be highly active (Zecca et al., 1996). Both membrane-tethered forms of Wingless were expressed under the control of the *ap-Gal4* driver and both were found to localise at the surface of expressing cells. In particular, Fig. 1J,N shows that NRT-HA[C93A]Wingless could be detected using the extracellular-staining protocol. Despite its localisation at the cell surface, NRT-HA[C93A]Wingless completely lacked signalling activity (adult flies did not show any phenotype and expression of *senseless*, a known target gene, was unaffected; not shown). This is in clear contrast to NRT-HA-Wingless, which is lethal at pupal stages because of the strong activation of target genes (Struhl and Basler, 1993). Therefore, restoring membrane localisation to [C93A]Wingless does not rescue signalling activity. We conclude that the palmitate moiety is strictly required for signalling activity, either for engagement with its receptor or to induce the appropriate conformational changes within the receptor upon binding.

[S239]Wingless and [C93A]Wingless are secreted by S2 cells

The absence of detectable HA-[C93A]Wingless at the surface of imaginal discs suggests that palmitoylation could be essential for secretion in vivo. Alternatively, as suggested above, [C93A]Wingless could be secreted but would not be detectable

outside the expression domain because of lack of retention at the cell surface and rapid dilution in the extracellular space. Willert et al. (Willert et al., 2003) favour the latter explanation because they found that palmitoylation-deficient Wnt3a is secreted by mammalian cells in culture. To measure the secretion of Wingless variants by *Drosophila* S2 cells, we generated fusion proteins with firefly luciferase, measured the amount of luciferase activity in the medium and normalised it to the total amount of luciferase activity that was produced. We found that luciferase-[C93A]Wingless as well as luciferase-[S239]Wingless are secreted into the medium to the same extent as luciferase-Wingless (Fig. 2A). Therefore, we conclude that lipid modifications are not essential for secretion of Wingless from *Drosophila* cells in culture.

Palmitoylation of Wingless at C93 promotes binding to Frizzled 2

As shown above, [C93A]Wingless had no signalling activity, even when it was forced to accumulate at the cell surface with an exogenous transmembrane domain. This could be because [C93A]Wingless has a reduced affinity for Fz2. No direct quantitative assay is yet available to measure the binding of Wingless to full-length Fz2. As an alternative, we adapted a paracrine signalling assay to infer binding information. In this assay, cells expressing HA-Wingless or HA-[C93A]Wingless were co-cultured with cells carrying a reporter plasmid, a Fz2-expressing plasmid and a plasmid expressing Histone-GFP. The latter construct was included so that receiving cells could be distinguished from Wingless-producing cells by fluorescence microscopy. Measurements of reporter activity with luciferase assays confirmed that HA-[C93A]Wingless does not trigger signalling, whereas HA-Wingless does (Fig. 2B). Furthermore, we confirmed that wild-type Wingless accumulates within non-expressing receiving cells (Fig. 2C,E), presumably following Fz2-mediated endocytosis (Piddini et al., 2005). By contrast, HA-[C93A]Wingless could not be detected within receiving cells (Fig. 2D,F). One likely interpretation is that HA-[C93A]Wingless does not bind Fz2 and hence cannot be internalised.

We next investigated whether an interaction between Fz2 and [C93A]Wingless could be detected when these proteins are expressed in the same cells. This is a less stringent assay because it does not require the ligand to travel in the extracellular medium in order to find its receptor on non-expressing cells. We found that co-overexpression of Fz2 and [C93A]Wingless leads to some accumulation of [C93A]Wingless at the cell surface in S2 cells (not shown). This is also the case in imaginal discs. As shown in Fig. 2G-J, overexpression of Flag-tagged Fz2 renders HA-tagged [C93A]Wingless detectable by extracellular staining. One possibility is that exogenous Fz2 could help [C93A]Wingless overcome a block in secretion, perhaps escorting it to the cell surface. Alternatively, [C93A]Wingless could independently reach the cell surface and be retained by Fz2. In either case, the above results suggest that Fz2 can interact (directly or indirectly) with [C93A]Wingless but to a much weaker extent than with wild-type Wingless. Such reduced affinity could account for the loss of signalling activity.

Retention of [C93A]Wingless in the ER of imaginal disc cells

The absence of [C93A]Wingless from the cell surface suggested an impairment in secretion. To further investigate this possibility, we turned to comparative immunofluorescence and immuno-EM. First, we set out to compare the localisation of wild-type Wingless and [C93A]Wingless by co-expressing these two forms, each with a distinct tag (HA for [C93A]Wingless and GFP for wild-type Wingless), and comparing the distribution by double immunofluorescence. For this, we used the epidermal cells of third-instar larvae because they are polyploid and hence large. As a control, HA-Wingless and GFP-Wingless (both wild type) were co-expressed. As expected, the subcellular localisation of the two tags (both reporting wild-type Wingless) was found to be undistinguishable (Fig. 3A-C). By contrast, expression of wild-type and [C93A]Wingless only partially overlapped in expressing epidermal cells (Fig. 3D-F). Note, in particular, the absence of [C93A]Wingless in large structures, possibly multivesicular bodies (MVBs), in which wild-type Wingless is present (arrowheads, Fig. 3D-F). In order to quantify this observation, we counted the number of vesicles that had GFP signal alone (wild-type Wingless), i.e. no HA signal (either [C93A]Wingless or wild-type for control). This was done in three different cells (three regions of interest each). We found threefold more single-labelled vesicles when HA-[C93A]Wingless and GFP-Wingless were co-expressed (34.6±7.9; n=9) than when HA-Wingless and GFP-Wingless were co-expressed (12.3±4.06; n=9). This was statistically significant ($P \geqslant 0.000118$). Therefore, we conclude that the absence of the palmitate moiety affects Wingless trafficking, although we cannot, at the light-microscope resolution, distinguish between effects on endocytosis or secretion.

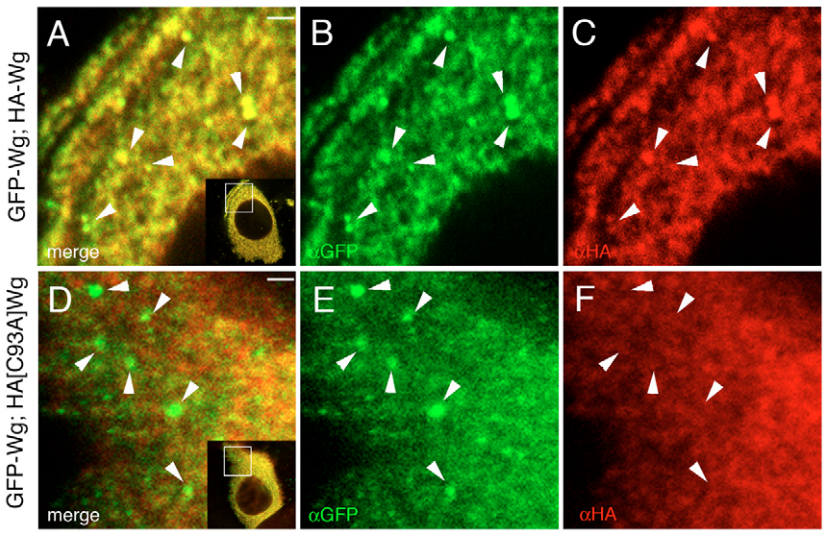

Fig. 3. Subcellular localization of wild-type Wingless and [C93A]Wingless in ectodermal cells of third-instar larvae. (A-C) High-magnification view of a region of ectodermal cells co-expressing wild-type Wingless from two transgenes (one tagged with GFP and the other with HA) with the *wg-Gal4* driver. Inset in A shows the whole cell. GFP is shown in green and HA in red. Extensive overlap is seen (yellow in A), including in large structures (arrowheads). (D-F) Co-expression of GFP-tagged wild-type Wingless (green) with HA-tagged [C93A]Wingless (red). Again, the whole cell is shown in the inset in D. Only partial overlap is seen between the mutant and wild-type proteins (D). Note, for example, the lack of colocalisation in large structures (arrowheads). Scale bars: 1 μm.

In order to characterise the trafficking differences between wild-type and [C93A]Wingless at the ultrastructural level, we turned to immuno-EM of imaginal disc cells. Strikingly, the overall number of gold particles in [C93A]Wingless-expressing cells was significantly higher than in cells expressing wild-type Wingless (Fig. 4A-D) even though the overall level of expression was the same for the two transgenes. Much of the [C93A]Wingless was in the ER (Fig. 4A,C). Indeed, quantitative analysis of the number of gold particles per unit of ER-membrane length revealed that [C93A]Wingless is present in the ER at a tenfold higher density than wild-type Wingless (compare overall staining in Fig. 4A,C and B,D). Wild-type Wingless was also enriched in the ER; however, it was consistently found at the plasma membrane, and particularly in MVBs (asterisk in Fig. 4G,H). In particular, despite showing frequent labelling for wild-type Wingless, MVBs were mostly devoid of [C93A]Wingless (asterisk in Fig. 4E,F). Because palmitate-deficient Wingless accumulates inside expressing cells, we conclude that, in vivo, the palmitate moiety is required for secretion. Most likely, the palmitate moiety is required for efficient transfer of Wingless from the ER to post-Golgi compartments.

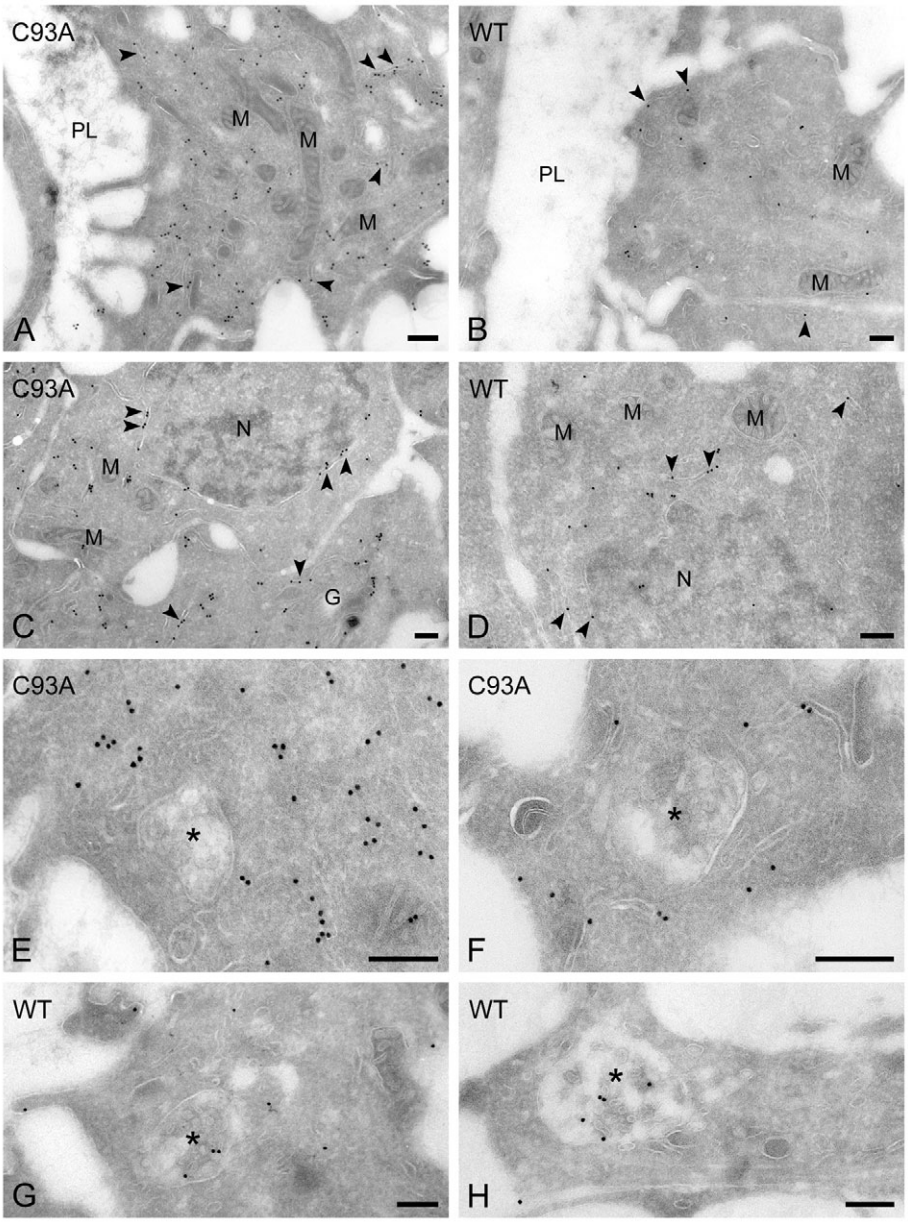

Fig. 4. [C93A]Wingless accumulates in the ER of wing disc cells and is absent from MVBs. (A,B) Portion of the apical surface of a disc cell. Empty space is the peripodial lumen (PL). HA-[C93A]Wingless and HA-Wingless (WT) expressed with *dpp-Gal4* are visualized with anti-HA and a secondary antibody labelled with 15-nm gold particles. Arrowheads indicate examples of HA-positive ER areas. These are particularly abundant in the [C93A]Wingless sample. (C,D) Basolateral region of disc cells. Note again the relatively abundant labelling in the perinuclear and other ER areas of cells expressing [C93A]Wingless (C). See for comparison an equivalent area in wild-type-Wingless-producing cells (D). Arrowheads indicate examples of ER areas positive for Wingless labelling. (E,F) MVBs (*) in cells expressing high levels of [C93A]Wingless. Note the absence of label in these areas. (G,H) By contrast, anti-HA labelling is seen in the MVBs of cells expressing wild-type Wingless. G, Golgi unit; M, mitochondrion; N, nucleus; PL, peripodial lumen. ER is recognised by its characteristic morphology, as indicated from an independent preparation stained with anti-KDEL (see supplementary material, Fig. S1). Scale bars: 200 nm.

Conclusion

Recent work with mammalian cell culture has suggested that the two lipid adducts appended to Wnt proteins might have specialised functions. Palmitate at C93 in Wnt1, at C77 in Wnt3a, C104 in Wnt5a or C93 in Wingless would be dedicated to signalling activity, whereas palmitoleic acid at S209 in Wnt3a or at S239 in Wingless would be essential for progression through the secretory pathway (Galli et al., 2007; Kurayoshi et al., 2007; Takada et al., 2006; Willert et al., 2003). With respect to secretion, we found that the role of lipids appears to be reversed in *Drosophila* Wingless, at least in vivo. We found that C93 is essential for progression through the secretory pathway, whereas S239 appears to be relatively dispensable in this function. We suggest, therefore, that the exact nature and position of the lipid adduct is not crucial for secretion and that different Wnts might use one or the other preferentially. Because [C93A]Wingless is secreted normally by S2 cells, it appears that the lipid requirement for secretion is somewhat relaxed in cultured cells, perhaps because they are not polarised. With respect to signalling, the overall level of lipidation clearly matters, because both C93 and S239 are essential for in vivo activity. Considering that palmitate at C93 is required both for binding to Fz2 and for progression through the secretory pathway, it is conceivable that this lipid moiety could mediate binding to Wntless (also known as Evi and Sprinter), another seven-pass transmembrane, which contributes to Wingless secretion (Banziger et al., 2006; Bartscherer et al., 2006; Goodman

et al., 2006). Further biochemical work is needed to assess whether and how lipid modification affects the binding of Wnts to these receptors.

Materials and Methods

Antibodies, plasmids and fly stocks

Primary antibodies used were mouse anti-Wingless 4D4 (prepared from cells obtained from the DSHB), mouse M2 anti-Flag (Sigma; 1/1000), mouse anti-HA 1.1 (Covance; 1/500), rabbit anti-HA (Y-11) (Santa Cruz Biotechnology; 1/1000), rabbit anti-GFP (Abcam; 1/2500). Secondary antibodies (all from Molecular Probes) were Alexa-Fluor-488-conjugated goat anti-rabbit (1/200), Alexa488-conjugated goat anti-mouse (1/200), Alexa594-conjugated goat and anti-mouse (1/200) and Alexa-Fluor-594-conjugated goat and anti-rabbit (1/200). Expression plasmids for in vitro studies were derived from pMT/V5-HisA (Invitrogen), which allows induction of expression with copper ions. Wingless fusion genes were generated by inserting DNA encoding HA or HA–firefly-luciferase into the unique *Pfl*MI site located near to the 5′ end of the wingless ORF. The C93A and S239A mutations were introduced using the QuikChange site-directed mutagenesis kit from Stratagene according to the manufacturer's manual and verified by sequencing. The same protocol was used to introduce the C93A mutation in NRT-HA-Wingless (Struhl and Basler, 1993). For expression in transgenic flies, the various constructs were transferred into the pUAST vector. We used fly stocks carrying the following transgenes: *UAS-HA-Wingless* and *UAS-NRT-HA-Wingless* (from G. Struhl, Columbia University, New York, NY), *UAS-flag-Fz2* (Piddini et al., 2005), *UAS-GFP-Wingless* (Pfeiffer et al., 2000), *ap-Gal4* and *dpp-Gal4*. *UAS-HA-[C93]Wingless, UAS-NRT-HA-[C93]Wingless* and *UAS-HA-[S239]Wingless* were generated for this study as described above. For co-overexpression of HA-Wingless and GFP-Wingless in larval ectodermal cells, we used *wg-Gal4* as a driver and Tub-Gal80^{TS} to prevent expression at embryonic stages, thus overcoming premature lethality. Larvae were switched to 29°C at the end of first instar to activate Gal4-dependent expression.

Immuno-EM

Larvae were inverted in Ringer solution and fixed in 2% PFA and 0.2% glutaraldehyde in 0.1 M phosphate buffer (PB), pH 7.4, for 3 hours at room temperature. After washing in PBS, larvae were kept in 1% PFA/PBS at 4°C. Subsequently, wing discs were dissected, embedded in 12% gelatin, mounted on aluminium pins and frozen in liquid nitrogen. Wing discs were then cut along the anteroposterior axis on a Reichert Ultracut S cryotome at –120°C. Antibody- and gold-labelling procedures were performed as described previously (Slot et al., 1991). 60-nm-thick cryosections were incubated with monoclonal anti-HA antibodies (clone 12CA5, Boehringer Mannheim). Anti-HA antibodies were detected with rabbit anti-mouse IgG (DakoCytomation Denmark A/S, Glostrup, Denmark) followed by protein A conjugated to 10- or 15-nm gold particles.

Cell culture, transfection and luciferase assay

S2 cells were cultured at 25°C in Schneider's complete medium supplemented with FCS to a 10% final concentration. The calcium phosphate transfection kit from Invitrogen was used according to the manufacturer's protocol. Cells were induced by overnight treatment with copper sulfate to a final concentration of 100 μM. A luminometer (TD-20/20) and the Promega Dual-Luciferase reporter assay system were used to measure the luciferase activities of firefly luciferase and *Renilla* luciferase. All the experiments were performed at least three times, and each time at least in triplicate.

This work was supported by the UK's Medical Research Council, the Endotrack programme of the European Union (F.W. and J.-P.V.) and the Dutch Cancer Society (grant number UU 2006-3508 to M.M.M.). We thank Cyrille Alexandre for help with transgenesis, Gary Struhl for plasmids and the Developmental Studies Hybridoma Bank for antibodies.

References

Banziger, C., Soldini, D., Schutt, C., Zipperlen, P., Hausmann, G. and Basler, K. (2006). Wntless, a conserved membrane protein dedicated to the secretion of Wnt proteins from signaling cells. *Cell* **125**, 509-522.

Bartscherer, K., Pelte, N., Ingelfinger, D. and Boutros, M. (2006). Secretion of Wnt ligands requires Evi, a conserved transmembrane protein. *Cell* **125**, 523-533.

Galli, L. M., Barnes, T. L., Secrest, S. S., Kadowaki, T. and Burrus, L. W. (2007). Porcupine-mediated lipid-modification regulates the activity and distribution of Wnt proteins in the chick neural tube. *Development* **134**, 3339-3348.

Goodman, R. M., Thombre, S., Firtina, Z., Gray, D., Betts, D., Roebuck, J., Spana, E. P. and Selva, E. M. (2006). Sprinter: a novel transmembrane protein required for Wg secretion and signaling. *Development* **133**, 4901-4911.

Greaves, S., Sanson, B., White, P. and Vincent, J. P. (1999). A screen for identifying genes interacting with armadillo, the *Drosophila* homolog of beta-catenin. *Genetics* **153**, 1753-1766.

Kurayoshi, M., Yamamoto, H., Izumi, S. and Kikuchi, A. (2007). Post-translational palmitoylation and glycosylation of Wnt-5a are necessary for its signalling. *Biochem. J.* **402**, 515-523.

Miura, G. I. and Treisman, J. E. (2006). Lipid modification of secreted signaling proteins. *Cell Cycle* **5**, 1184-1188.

Miura, G. I., Buglino, J., Alvarado, D., Lemmon, M. A., Resh, M. D. and Treisman, J. E. (2006). Palmitoylation of the EGFR ligand Spitz by Rasp increases Spitz activity by restricting its diffusion. *Dev. Cell* **10**, 167-176.

Pepinsky, R. B., Zeng, C., Wen, D., Rayhorn, P., Baker, D. P., Williams, K. P., Bixler, S. A., Ambrose, C. M., Garber, E. A., Miatkowski, K. et al. (1998). Identification of a palmitic acid-modified form of human Sonic hedgehog. *J. Biol. Chem.* **273**, 14037-14045.

Pfeiffer, S., Alexandre, C., Calleja, M. and Vincent, J. P. (2000). The progeny of wingless-expressing cells deliver the signal at a distance in Drosophila embryos. *Curr. Biol.* **10**, 321-324.

Piddini, E., Marshall, F., Dubois, L., Hirst, E. and Vincent, J. P. (2005). Arrow (LRP6) and Frizzled2 cooperate to degrade Wingless in Drosophila imaginal discs. *Development* **132**, 5479-5489.

Slot, J. W., Geuze, H. J., Gigengack, S., Lienhard, G. E. and James, D. E. (1991). Immuno-localization of the insulin regulatable glucose transporter in brown adipose tissue of the rat. *J. Cell Biol.* **113**, 123-135.

Strigini, M. and Cohen, S. M. (2000). Wingless gradient formation in the Drosophila wing. *Curr. Biol.* **10**, 293-300.

Struhl, G. and Basler, K. (1993). Organizing activity of wingless protein in Drosophila. *Cell* **72**, 527-540.

Takada, R., Satomi, Y., Kurata, T., Ueno, N., Norioka, S., Kondoh, H., Takao, T. and Takada, S. (2006). Monounsaturated fatty acid modification of Wnt protein: its role in Wnt secretion. *Dev. Cell* **11**, 791-801.

Tanaka, K., Kitagawa, Y. and Kadowaki, T. (2002). Drosophila segment polarity gene product porcupine stimulates the posttranslational N-glycosylation of wingless in the endoplasmic reticulum. *J. Biol. Chem.* **277**, 12816-12823.

Willert, K., Brown, J. D., Danenberg, E., Duncan, A. W., Weissman, I. L., Reya, T., Yates, J. R., 3rd and Nusse, R. (2003). Wnt proteins are lipid-modified and can act as stem cell growth factors. *Nature* **423**, 448-452.

Zecca, M., Basler, K. and Struhl, G. (1996). Direct and long-range action of a wingless morphogen gradient. *Cell* **87**, 833-844.