Roles of N-glycosylation and lipidation in Wg secretion and signaling

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Wnt members act as morphogens essential for embryonic patterning and adult homeostasis. Currently, it is still unclear how Wnt secretion and its gradient formation are regulated. In this study, we examined the roles of N-glycosylation and lipidation/acylation in regulating the activities of Wingless (Wg), the main Drosophila Wnt member. We show that Wg mutant devoid of all the N-glycosylations exhibits no major defects in either secretion or signaling, indicating that N-glycosylation is dispensable for Wg activities. We demonstrate that lipid modification at Serine 239 (S239) rather than that at Cysteine 93 (C93) plays a more important role in regulating Wg signaling in multiple developmental contexts. Wg S239 mutant exhibits a reduced ability to bind its receptor, Drosophila Frizzled 2 (dfz2), suggesting that S239 is involved in the formation of a Wg/receptor complex. Importantly, while single Wg C93 or Wg S239 mutants can be secreted, removal of both acyl groups at C93 and S239 renders Wg incapable of reaching the plasma membrane for secretion. These data argue that lipid modifications at C93 and S239 play major roles in Wg secretion. Further experiments demonstrate that two acyl attachment sites in the Wg protein are required for the interaction of Wg with Wntless (Wls), also known as Evi or Srt), the key cargo receptor involved in Wg secretion. Together, our data demonstrate the in vivo roles of N-glycosylation and lipid modification in Wg secretion and signaling.

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Introduction

The Wnt family members comprise an evolutionarily conserved class of secreted signaling molecules which control growth and patterning in various developmental contexts and maintenance of tissues in adult homeostasis (Cadigan and Liu, 2006; Cadigan and Peifer, 2009; Clevers, 2006; Logan and Nusse, 2004). De-regulated Wnt signaling has been implicated in a variety of developmental abnormalities and cancers (Logan and Nusse, 2004; MacDonald et al., 2009; Polakis, 2007). Normal cells require an orchestrated program of actions to tightly and precisely control Wnt activities. Various studies have shown that post-translational modifications of Wnt proteins play critical roles in many aspects of Wnt regulation, including ligand secretion, extracellular distribution and receptor activation. However, it is less clear about the roles of post-translational modifications in regulating Wnt activities in specific developmental contexts.

As a common structural characteristic, Wnts contain a high number of conserved cysteines (23–25 on average), suggesting that the formation of intra- and inter-molecular disulfide bonds may be important for the proper folding and multimerization of Wnt proteins. In addition to disulfide bonding, two major types of post-translational modifications, lipidation/acylation and N-glycosylation, have been reported on most Wnt members. It has been shown that Drosophila Wingless (Wg), murine Wnt1, Wnt3a and Wnt5a, as well as chick Wnt1 and Wnt3a are all palmitoylated at the first conserved cysteine residue (C93 in Wg) (Doubravska et al., 2011; Galli et al., 2007; Kurayoshi et al., 2007; Miura and Treisman, 2006; Willett et al., 2003). Wnt3a has been reported to be lipid-modified by palmitoleic acid at a second site, serine 209, which is also conserved among Wnt members (S239 in Wg) (Takada et al., 2006). Therefore, two acyl groups can be attached to Wnts: one palmitate at an N-terminal cysteine and one palmitoleic acid at an internal serine. The only exception known so far is WntD, a Drosophila Wnt family member which does not have the conserved serine and does not undergo any lipid modification (Ching et al., 2008). In vertebrates, studies from cell-based assays about the role of lipidation argued that palmitate at cysteine is essential for Wnt signaling (Galli et al., 2007; Kurayoshi et al., 2007; Miura and Treisman, 2006; Willett et al., 2003; Krumlauf et al., 2003).
while palmitoleic acid at serine is required for Wnt secretion (Takada et al., 2006). However, it has been recently reported that in several cellular contexts, murine Wnt1 and Wnt3a lacking the cysteine-linked palmitate can still signal (Doubrovskova et al., 2011).

Many lines of evidence strongly argue that Wnt lipid modification is controlled by the endoplasmic reticulum (ER) protein Porcupine (Porc). Porc encodes a conserved multiple-pass transmembrane protein in the family of membrane-bound O-acyltransferases (MBOATs) (Hofmann, 2000). Porc loss-of-function mutations phenocopy mutations of Wnt acylation and show similar disrupted secretion of Wnt3a (Takada et al., 2006; van den Heuvel et al., 1993). After post-translational modifications, mature Wnt proteins exit from the ER and are secreted in a pathway that requires the function of the carrier protein, Wls (Banziger et al., 2006; Bartscherer et al., 2006; Goodman et al., 2006). Wls is a multi-pass transmembrane protein and has been shown to be localized in the ER, Golgi apparatus and on the plasma membrane (Banziger et al., 2006; Bartscherer et al., 2006; Belenkaya et al., 2008; Coombs et al., 2010; Yang et al., 2008). After released from the cell surface, Wnt molecules reach receiving cells by a facilitated movement involving lipoprotein particles and heparan sulfate proteoglycans (HSPG, Dally and Dlp in Drosophila) (Baeg et al., 2001; Franch-Marro et al., 2005; Han et al., 2004, 2005; Lin, 2004; Neumann et al., 2009; Panakova et al., 2005).

Various studies have indicated that Wnts can be N-glycosylated, in which multiple asparagine residues of Wnts are appended with N-linked oligosaccharide chains. Unlike lipid modification, different Wnt members vary in the number and position of glycosylation sites and the roles of glycosylation also seem to be unconserved within the Wnt family. Particularly, it has been demonstrated that replacement of all N-glycosylation sites does not affect Wnt1-induced autocrine or paracrine signaling in several cellular contexts, indicating that glycosylation is not essential for either secretion or signaling (Doubrovskova et al., 2011; Mason et al., 1992). However, in the case of Wnt3a and Wnt5a, the Kikuchi group argued that glycosylation precedes lipid modification and is important for Wnt secretion but not for Wnt actions (Komekado et al., 2007; Kurayoshi et al., 2007).

Most available data regarding the activities of Wnt lipidation and N-glycosylation were obtained from cell-based assays (Komekado et al., 2007; Kurayoshi et al., 2007; Takada et al., 2006; Willert et al., 2003). As a common practice, Wnt members and its mutant derivatives are usually examined by using specific epitope tags which may complicate the interpretation of results. Thus, to fully understand the respective functions of Wnt lipidation and glycosylation, we need to evaluate their roles by using non-tagged proteins and examining their in vivo activities in specific developmental contexts.

In the present study, we aim to investigate how N-glycosylation and lipidation contribute to Wg signaling and secretion using Drosophila embryos and wing imaginal discs as in vivo systems. During embryonic and wing development, Wg acts as both a short-range inducer and a long-range morphogen to regulate tissue patterning (Clevers, 2006; Kohn and Moon, 2005). After release from its origin, Wg forms a graded distribution throughout the area of receiving cells where it binds to the receptors of the Frizzled family (mainly Frizzled_2, dFz2) to activate downstream signaling. In this paper, we generated Wg mutant variants defective in lipidation or glycosylation and analyzed their signaling properties in embryos and wing imaginal discs. Our data show that glycosylation-deficient Wg can be secreted and still maintains major signaling activity. However, although palmitate at C93 is not absolutely required for secretion or signaling, palmitoleic acid at S239 contributes significantly to signaling activity. Importantly, our results indicate that Wg binding to Wls requires at least one of the two lipid adducts and that loss of dual lipidation disrupts Wg–Wls interaction, thereby disabling Wg secretion through the dedicated secretory route. Our results also clarified some controversial issues in previous functional studies about the roles of lipida

### Materials and methods

**Wg transgenes and constructs**

The C93A, S239A and N103Q mutations were introduced into full-length Wingless using the GeneTailor™ site-directed mutagenesis kit from Invitrogen and were verified by sequencing. The C93A mutation was also introduced to UAS-HA_Wg (from Gary Struhl) using the same protocol. The N414S mutation was cloned from the construct pCaspeR hs WgN414S (Tanaka et al., 2002). All constructs were cloned into the pUAST vector for expression in transgenic flies. WgK22 (van den Heuvel et al., 1993) is used as Wingless null allele and UAS-WgE3 (Brennan et al., 1999) is used for over-expression of wild-type Wingless.

**Fly genetics**

Ectopic expression of Wg transgenes was achieved by the Gal4/UAS system (Brand and Perrimon, 1993). daGal4, hhGal4, dppGal4, apGal4, and simGal4 strains were described in Flybase. y w flp; Act> y + > Gal4 UAS-GFP (Ito et al., 1997) was used to induce the expression of Wg transgenes in GFP-marked random clones in the wing discs. To achieve co-expression of Ws-V5 with Wg or WgCS in the wing discs, UAS-Wg transgene/wt-Wls-V5; hhGal4 tub1a-Gal80D larvae were kept at 18 °C until third instar to block Gal4, then shifted to 30 °C to allow Gal4 expression, heat-shocked for 2 h at 37 °C to induce Wls-V5 expression and kept at 30 °C for the rest of time (about 3–4 h) before dissection. To ectopically express Wg transgenes (or arm.S10) in Wg null background, simGal4 wggK22/GycyctkGP recombinants were generated and crossed with wggK22; UAS-Wg transgenes/(or UAS-arm.S10)/T(2;3)Sm6a-TM6B. In the next generation, embryos with the desired hetero-allelic genotypes (simGal4 wggK22/wggK22, UAS-Wg transgenes/+ ) were identified without ambiguity by excluding GFP embryos. UAS-arm.S10 was described in Flybase.

**Imaginal disc and embryo immunostaining**

Fixation and antibody staining of imaginal discs and embryos were performed as described (Belenkaya et al., 2002, 2008). Fixation and antibody staining in cultured cells were performed as described (Belenkaya et al., 2008). Extracellular Wg staining was performed as described (Strigini and Cohen, 2000). Polyclonal guinea pig anti-Wg antibody was generated against a CBP fusion protein of Wg corresponding to amino acid residues 258–367 of the whole protein. Other primary antibodies used include mouse anti-Wg 4D4 (Iowa Developmental Studies Hybridoma Bank; IDSBH), guinea pig anti-Neuroglass (Nolo et al., 2000), rabbit anti-Fz2 (Mathew et al., 2005), rabbit anti-Hrs (Santa Cruz), rabbit anti-Giantin (Covance), rabbit anti-Calnexin (Stressgen) and mouse anti-V5 (Invitrogen).

**Luciferase assay**

Drosophila S2 cells were used in the luciferase reporter assay. Transfections were performed in 24-well plates using Effectene transfection reagent (QIAGEN). For autocrine activation luciferase assay, TOPFlash-like luciferase reporter dTF2 (DasGupta et al., 2005) and the normalization vector PoIIIIRL (40:1 ratio) were transfected along with plasmids expressing dFz2 and equal amounts of plasmids expressing different Wingless variants respectively. 48 hours later, cells were lysed and luciferase activities were measured using Dual-Luciferase Assay Kits (Promega). For paracrine activation luciferase assay, 'the donor cells' were co-transfected with different Wg expression vectors and PoIIIIRL normalization vectors while 'the receiving cells' were co-transfected with dTF2 and a plasmid encoding dFz2. 40 hours later, 'the donor cells' and 'the receiving cells' were mixed together and co-cultured for another 24 h before the luciferase activities were measured.
**Results**

**N-glycosylation is dispensable for Wg signaling and secretion**

Wg protein was reported to have two major N-glycosylation sites, Asn103 and Asn414 (N103 and N414) (Tanaka et al., 2002). To examine the in vivo functions of these two N-glycosylation sites, we generated Wg mutants containing single or double amino acid substitutions, WgN103Q (N103 converted to Gln), WgN414S (N414 converted to Ser) and WgNN (combination of N103Q and N414S) (Supplementary Fig. 1A). Consistent with previous report, while wild-type Wg exhibits two additional glycosylated bands in Western blots, WgNN was detected as a single non-glycosylated band in both transfected S2 cells and in wing disc cells (Supplementary Figs. 1B–D), indicating that Wg is normally glycosylated at N103 and N414.

We first examined the activities of Wg variants in cultured Drosophila S2 cells by luciferase assays. To trigger autocrine Wg signaling, S2 cells were co-transfected with a dFz2-expressing plasmid, a luciferase reporter plasmid and expression constructs encoding either wild-type Wg or mutant Wg proteins. To activate paracrine Wg signaling, ‘donor cells’ expressing different Wg variants were co-cultured with ‘receiving cells’ transfected with a dFz2-expressing plasmid and a luciferase reporter plasmid. Glycosylation-deficient Wg variants can activate downstream signaling in both assays (Figs. 1A and B) although a relatively mild reduction was observed when compared to wild-type Wg (reduced by 20% for autocrine signaling and 40% for paracrine signaling).

To further evaluate the in vitro observations, we generated transgenic flies expressing Gal4-inducible wild-type Wg or various Wg mutants deficient in certain post-translational modifications. All transgenic lines express comparable levels of Wg proteins, which is confirmed by Western blots and immuno-staining (data not shown). Consistent with the data from cell culture, three lines of evidence suggest that N-glycosylation is not essential for Wg signaling in multiple developmental contexts. First, Wg proteins deficient in single or double glycosylations are capable of patterning embryonic epidermis via autocrine signaling. The ventral cuticles of wild-type embryos are characteristic of a repeated pattern of denticle belts interspersed by naked cuticles (Fig. 1C). It has been shown that activation of the Wg pathway is sufficient for the formation of naked cuticles (Lawrence et al., 1996; Noordermeer et al., 1994). As expected, daughterless-Gal4 (daGal4)-driven over-expression of wild-type Wg resulted in replacement of ventral belts with naked cuticles (Fig. 1D). Similar cuticle phenotypes were obtained with daGal4-driven expression of all three glycosylation mutants (Figs. 1G–I). Since daGal4 is ubiquitously expressed in the embryos (Wodarz et al., 1995), this result indicates that non-glycosylated Wg is active in Wg autocrine signaling. Second, under more stringent conditions, we examined Wg paracrine signaling induced by different Wg variants in embryonic cuticle patterning. single minded (sim) is a gene specifically expressed in the midline neuroepithelium of the embryos (Thomas et al., 1988) (Fig. 1K). simGal4-driven expression of wild-type Wg can rescue the formation of naked cuticles in the ventral ectoderm of Wg-null embryos (Fig. 1N). Here paracrine Wg signaling is required for the rescuing effect as Wg ligands need to act cell-non-autonomously in the neighboring tissue. As such, armadillo.S10 (arm.S10) which induces cell-autonomous constitutively-active Wg signaling independent of a Wg ligand (Pai et al., 1997), failed to restore naked cuticle formation (Fig. 1M). However, the unglycosylated Wg, WgNN, retained a reduced yet significant degree of rescuing capacity which was reflected by a smaller range of naked cuticles formed in the ventral midline (Fig. 1Q). Third, we compared the ability of wild-type and mutant Wg proteins to induce downstream gene expression in the wing imaginal discs. In the third-instar larval wing disc, Wg is produced in a narrow strip of cells along the dorsoventral (DV) border. In regions proximal to the border, the short-range target Senseless (Sens) is expressed (Figs. 2A and B–B″). In this analysis, WgNN displayed signaling activity similar to wild-type Wg, demonstrated by strong induction of ectopic Sens expression (Figs. 2C–C″ and F–F″). Taken together, the above results suggest that N-glycosylation is dispensable for Wg secretion and signaling.

**S239, but not C93, is required for Wg signaling**

Using similar assays, we also examined the effects of lipidation on Wg signaling activities. Single acyl-deficient Wg was made by replacement of the essential residues with alanine; Cys93 to Ala in WgC93A and Ser239 to Ala in WgS239A, respectively (Supplementary Fig. 1A). Both lipidation mutants are produced and modified normally as they show glycosylation patterns similar to that of the wild-type counterpart (Supplementary Fig. 1B). In cultured S2 cells, only a mild reduction of luciferase activities was observed in WgC93A in both autocrine and paracrine assays (Figs. 1A and B). Similarly, WgS239A was able to induce the formation of naked cuticles in the embryos via cell-autonomous signaling (Fig. 1E) and cell-non-autonomous signaling (Fig. 1O). Furthermore, in wing discs, WgC93A functioned to stimulate Sens expression similar to that in wild-type Wg (Figs. 2C–D″). Together, these data demonstrate that WgC93A still maintains major Wg signaling activities, suggesting that palmitoylation at C93 is not essential for Wg signaling.

Importantly, we found that in contrast, WgS239A mutants showed poor signaling activities. In S2 cells, WgS239A acts weakly to stimulate the Wg luciferase reporter in both autocrine and paracrine activation assays (Figs. 1A and B). Consistent with these data, in the embryos, expression of WgS239A was not able to activate the downstream signaling required for the formation of naked cuticles (Figs. 1F and P). Further analysis in the wing discs revealed only residual signaling activity of this mutant as reflected by the minimal levels of ectopic Sens induction (Figs. 2E–E″).

Taken together, both in vitro and in vivo results argue that S239, but not C93 is important for Wg signaling.

**WgS239A has reduced affinity for Frizzled 2 receptor**

As shown above, WgS239A displayed markedly reduced signaling activities. Next, we examined the mechanisms underlying these signaling defects. One hallmark for normal Wg signaling is Wg punctuates in the receiving cells due to endocytosis following receptor interaction (Figs. 3A–A″). The absence of such punctuate structures
in cells surrounding S239A-expressing clones (Figs. 3C–C′) implies one possibility that Wg lacking the critical serine cannot be secreted normally and therefore fails to reach the cell surface to initiate downstream signaling, similar to the serine mutant of Wnt3a (Takada et al., 2006). To test this, we employed an extracellular staining protocol which only detects proteins on the cell surface and in the extracellular matrix. Following protein expression in the dorsal compartment of the wing discs by apGal4, we assessed the pool of secreted Wg by the extracellular staining and the pool of total Wg by conventional staining (Figs. 3E–E″). HA-tagged WgC93A, which has been shown to be retained in the ER (Franch-Marro et al., 2008), was included as a negative control. As expected, no signal of HA-WgC93A was detected by extracellular staining (Figs. 3G–G′). Contrary to this, we observed similar extracellular accumulation of wild-type Wg and WgS239A in the dorsal compartment (Figs. 3E–F″). In a second experiment, we examined secreted WgS239A protein in S2 cells. WgS239A could be retrieved from both the cell lysate and the conditioned medium of transfected S2 cells to the same extent as wild-type Wg and WgC93A mutant (Fig. 3H). Together, these data strongly suggest that WgS239A is still able to progress through the secretory pathway and be secreted from its producing cells.

As WgS239A secretion is not blocked, we ask whether the poor action of WgS239A is due to its disrupted interaction with dFz2 receptors. We adapted two assays to examine Wg-dFz2 interaction. First, we conducted a co-immunoprecipitation (co-IP) experiment to examine the complex formation between WgS239A and dFz2. We co-expressed full-length V5-tagged dFz2 with WgC93A, WgS239A or its wild-type counterpart in S2 cells. The portion of dFz2 interacting with Wg was detected by Western blotting in the immunoprecipitate of Wg from the cellular lysate. We observed a dramatic reduction in dFz2 binding when S239 is mutated, while some reduction was also found in WgC93A mutant (Fig. 4A and quantification in Fig. 4B). We further examined the interaction of Wg and WgS239A with dFz2 using a cell-labeling assay. In this assay, S2 cells transfected with a
dFz2-expressing plasmid were split into three samples, each of which was incubated for a short period with similar amounts of wild-type Wg, WgC93A or WgS239A respectively (confirmed by Western blots shown in Fig. 4F). Cells were alive and minimal endocytosis could occur as samples were kept in ice-cold water during incubation. Expression of dFz2 caused accumulation of exogenous Wg on the cell surface, which was revealed by extracellular staining. Representative data were shown in Figs. 4C–E″ and quantified in Fig. 4G. Consistent with co-IP results, similar levels of dFz2 trapped considerably more wild-type Wg than WgS239A mutant (Figs. 4B–C″). Although we cannot exclude other roles of S239 in Wg signaling, our data presented here suggest that acylation of Wg at S239 is involved in promoting binding to the dFz2 receptor.

Removal of double lipidation abolishes Wls-dependent Wg secretion

The observation that both WgC93A and WgS239A can be secreted normally led us to test the secretion and signaling of the double-acyl-deficient Wg, WgC93AS239A (WgCS). WgCS failed to signal in both S2 cells and in embryos (Figs. 5A and B). Similarly, in the wing disc, the signaling activity of WgCS was also completely abolished (Figs. 5C–C″). Importantly, we found that WgCS failed to reach the surface of the wing disc cells and cultured S2 cells when examined by the extracellular staining protocol (Figs. 6A–D″). Consistently, WgCS was absent from the medium of the S2 cells transfected with the expression construct (Fig. 6E). Taking all of these data together, we conclude that lipidation of Wg is essential for its secretion, and single lipidation at either C93 or S239 is sufficient to fulfill this requirement.

In light of the essential role of Wls in Wg secretion, we next tested whether reduction of lipidation alters the interaction between Wls and Wg. In wing disc cells co-expressing wild-type Wg and V5-tagged Wls, these two proteins co-localized well in vesicular structures (Figs. 7A–A″), possibly representing secretory vesicles. In addition, co-expression of wild-type Wg caused an apical redistribution of Wls (Fig. 7A′) (Port et al., 2008). In contrast, WgCS shows reduced co-localization with Wls and fails to redistribute Wls (Figs. 7D–D″), providing another piece of evidence for impaired interaction. The physical interaction between Wg and Wls was further tested in S2 cells by co-IP assay. Western blot analysis revealed that while normal binding to Wls maintained after removal of a single lipid modification, a double lipidation deficiency abolished the Wg–Wls interaction (Fig. 7E and F). Therefore, our study suggests that the overall
lipidation levels at C93 and S239 are required for the interaction of Wg with Wls, and therefore for Wg secretion.

**Discussion**

Lipidation and N-glycosylation are widely found in many Wnt members. In this paper, we systematically analyzed the in vivo roles of all known post-translational modifications of Wg. The principal findings of this study include: (1) Wg mutant devoid of all N-glycosylations exhibits no major defects in either secretion or signaling; (2) lipidation at S239 but not at C93 is critical for Wg signaling; (3) while WgC93A and WgS239A mutants can be secreted, removal of both acyl groups renders Wg incapable of interacting with Wls, resulting in its secretion defect.

**Roles of N-glycosylation in Wg signaling**

Consistent with the recent work on mouse Wnt1 (Doubravská et al., 2011), un-glycosylated Wg in our assays can be readily secreted and can actively signal in cultured cells and in various developmental
contexts. Although we did observe some reduction in signaling compared to wild-type Wg, the fact that \textit{wg\textsuperscript{Gal4}}-driven expression of WgNN in \textit{wg} mutant background can significantly restore naked cuticle formation strongly argues that WgNN is fairly functional (Supplementary Fig. 3F). Previous studies demonstrated that glycosylation deficient Wnt3a and Wnt5a are defective in secretion (Komekado et al., 2007; Kurayoshi et al., 2007). Thus our results, along with the previous study on Wnt1, strongly suggest that the involvement of glycosylation in secretion is not a common mechanism among different Wnt members. It is important to note that Wg glycosylation patterns are virtually identical among wild-type Wg, WgC93A, WgS239A and WgCS (Figs. 3H and 6E), indicating that Wg can be glycosylated even in the absence of lipidation at C93 and S239. On the other hand, unglycosylated Wg probably undergoes normal lipid modification, as a deficiency in lipidation of this Wg mutant would be expected to cause signaling and/or secretion defects. Thus, based on our data and other studies on Wnt1, we suggest that lipidation and N-glycosylation are likely to be two independent processes, at least for the modifications which occur at sites examined in this paper.

\textbf{Roles of lipidation in Wg signaling}

Previous work suggests that palmitate at the conserved cysteine is dedicated to signaling activity by affecting the affinity for Frizzled receptors (Franch-Marro et al., 2008; Galli et al., 2007; Komekado et al., 2007; Kurayoshi et al., 2007), while palmitoleic acid at the conserved serine is dedicated to secretion (Takada et al., 2006). Here we show that Wg lipidation at S239, but not at C93 appears to be important for Wg signaling. Our results also show that Wg secretion is impaired only when both sites are mutated. The differences between our results and the previous data suggest that the similar role in Wnt signaling (to promote Frizzled interaction), the nature and site(s) of lipid modification might be flexible among different Wnt members. In addition, a recent study on mouse Wnt1 and Wnt3a suggests that lipidation at the essential serine is a prerequisite for the subsequent lipid attachment at the cysteine residue (Doubravska et al., 2011). However, based on the different behaviours of WgC93A, WgS239A and WgCS, our data suggest that lipid modifications at these two sites are mutually independent.

\begin{figure}[h]
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\caption{The weaker binding of WgS239A with dFz2 receptor. (A) Co-IP assay. S2 cells were transfected with plasmids encoding wild-type Wg (or WgS239A) and V5-tagged dFz2 (dFz2-V5). Cell lysates were immunoprecipitated and then analyzed by Western blotting with the antibodies indicated. IP, immunoprecipitation; IB, immunoblot. (B) Quantification of the intensity of the co-precipitated dFz2 bands from co-IP assay in A. The relative intensity (ratio of mutants to wild-type) is shown as mean±s.d. (n=3, *P<0.05, **P<0.01 and ***P<0.001, t-test). (C–E) Cell labeling assay. S2 cells transfected with a dFz2-V5 expression vector were equally split into three samples. Each sample was incubated with the conditioned medium containing similar amount of wild-type Wg (C–C') or WgC93A (D–D') or WgS239A (E–E'). Wg proteins trapped on the cell surface by dFz2 were determined by Wg staining. (F) Amount of Wg variants in the conditioned media in the cell labeling assay was determined by Western blotting. Similar protein levels were detected. (G) Quantification of the Wg accumulation in cell labeling assay. Fluorescence intensities of Wg were normalized to the expression levels of dFz2 in individual cells. The relative dFz2 binding (ratio of mutants to wild-type) is shown as mean±s.d. (n=field of view)=5–7, *P<0.05, **P<0.01 and ***P<0.001, t-test).}
\end{figure}
We show that \textit{WgS239A} has a markedly reduced ability to interact with dFz2, suggesting that lipidation at S239 promotes binding to dFz2, thereby regulating Wg signaling activity. The impaired \textit{WgS239A–dFz2} interaction is the main mechanism underlying the signaling defects of \textit{WgS239A}, at least for its cell-autonomous action. It is also worth to note that the binding of \textit{WgC93A} with dFz2 is also significantly reduced, which might be one reason why this mutant is not as active as wild-type. It is still possible that the acyl group at S239 may regulate other aspects of the Wg pathway. For example, palmitoleic acid at S239 may act to facilitate binding to HSPGs and/or lipoproteins particles, which have been shown to help establish the Wg gradient (Baeg et al., 2001; Han et al., 2005; Lin, 2004; Panakova et al., 2005). Consistent with this, although WgS239A can reach the cell surface, it cannot spread as effectively as wild-type Wg to neighboring cells (Fig. 3F). Further studies are needed to determine other roles of S239 in Wg signaling.

**Fig. 5.** Removal of double lipidation abolishes Wg signaling activity. The signaling activities of WgCS were examined by the luciferase reporter in S2 cells. (A), cuticle patterning in embryos (B) and Sens induction in wing imaginal discs (C-C″). Same experiments were done as shown in Figs. 1 and 2.

**Fig. 6.** WgCS is retained in the producing cells. (A–B″) Extracellular distribution of wild-type Wg (A–A″) and WgCS (B–B″) in transfected S2 cells. (C–D″) Extracellular distribution of wild-type Wg (C–C″) and WgCS (D–D″) in wing discs. Expression of Wg variants in the wing disc was driven by \textit{dpGomp}. The membrane-associated WgCS was barely detectable in transfected cells and in wing disc cells. (E) Secretion of wild-type Wg and WgCS in S2 cells. Minimal amount of WgCS was present in the conditioned medium. A dsRed expression vector was co-transfected and blotted as a control for transfection efficiency.
Role of lipidation in Wg secretion

One important observation of this work is that removal of both lipid adducts at C93 and S239 sites renders Wg incapable of secretion. Importantly, we show that WgCS mutant exhibits a drastic reduction in its interaction with the Wls protein, arguing that a failure to interact with Wls might be a major mechanism underlying the secretion defect of WgCS in both cultured cells and the wing discs. However, the requirement of S239 for Wls recognition is somewhat different in vivo and in cultured cells: WgS239A interacts with Wls normally (Figs. 7E and F) and is secreted readily by S2 cells (Fig. 3H), while its colocalization with Wls and its effect on Wls relocalization are both defective in wing disc cells (Figs. 7C and C′), although it can still reach the cell surface (Fig. 3F). One possible explanation is that polarized cells may have a more stringent requirement for Wls recognition and Wg routing for secretion. Lipidation is probably a general requirement for Wnt binding to Wls as Wnt3A mutation at S209, Porcupine inhibition by either SiRNA or chemical inhibitors also lead to impaired Wnt–Wls interaction (Chen et al., 2009; Coombs et al., 2010). Intriguingly, recent work proposed a model that the first intraluminal loop of Wls contains a lipid-binding fold which is also present in the palmitate-binding lipocalin family of proteins (Coombs et al., 2010). Therefore, lipidation could be directly involved in the physical interaction of Wg with Wls. It appears that Wnts are designated to a dedicated secretory route which necessitates both the action of Wls and lipid modifications of Wnts. In support of this view, routing signal from the type I membrane protein Neurotactin could not shunt Wg to other Wls-independent secretory pathways (Banziger et al., 2006). In addition, a new Wnt member, WntD, which does not undergo any lipid-modification process, bypasses the dedicated secretion pathway and is secreted independent of Wls function (Ching et al., 2008).

HAWgC93A and WgC93Y show strong loss of function

Previous work demonstrated that a cysteine to alanine mutation at C93 impaired the signaling capacity of HA-tagged Wg and this HAWgC93A was defective in secretion in the wing disc cells but not in S2 cells (Franch-Marro et al., 2008). In contrast, we found that without any tag, WgC93A was considerably active in signaling in Droso-phila embryos, wing discs and cultured S2 cells. We have carefully re-examined the activity of HAWgC93A and confirmed the published data (Figs. 1A and J and Figs. 3G–G′). Thus, the unexpected discrepancy results from the HA tag insertion. In support of this view, we found that replacement of the HA tag by GFP in HAWgC93A can generate similar defects (data not shown). Together, these data suggest that...
insertion of specific protein tag such as HA and GFP at the N-terminal region of Wg might change its conformation and subsequently interfere with its activity. Another inconsistency in the literature comes from the wgS21 allele, which changes C93 into a tyrosine, is reported to be a strong loss of function allele (Willert et al., 2003). Indeed, when we examined WgC93Y in our systems, no signaling activities were detected in cultured cells (Supplementary Figs. 2A and B), embryos (data not shown) or wing discs (Supplementary Fig. 2E). Considering the remarkable signaling capacity of WgC93A, it is safe to conclude that while lipidation at C93 is not as important for protein function, a Cys to Tyr substitution may be deleterious for other reasons. Interestingly, another WgS239 mutant, WgS239D, behaves indistinguishably from WgS239A (Supplementary Figs. 2A, B, and F), further proving the necessity of lipidation at WgS239 for a functional protein. Thus, our data suggest that caution must be taken in functional studies when employing amino acid substitutions, and when introducing mutations in tagged proteins.

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References