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Lipid-independent Secretion of a *Drosophila* Wnt Protein*

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Wnt proteins comprise a large class of secreted signaling molecules with key roles during embryonic development and throughout adult life. Recently, much effort has been focused on understanding the factors that regulate Wnt signal production. For example, Porcupine and Wntless/Evi/Sprinter have been identified as being required in Wnt-producing cells for the processing and secretion of many Wnt proteins. Interestingly, in this study we find that WntD, a recently characterized *Drosophila* Wnt family member, does not require Porcupine or Wntless/Evi/Sprinter for its secretion or signaling activity. Because Porcupine is involved in post-translational lipid modification of Wnt proteins, we used a novel labeling method and mass spectrometry to ask whether WntD undergoes lipid modification and found that it does not. Although lipid modification is also hypothesized to be required for Wnt secretion, we find that WntD is secreted very efficiently. WntD secretion does, however, maintain a requirement for the secretory pathway component Rab1. Our results show that not all Wnt family members require lipid modification, Porcupine, or Wntless/Evi/Sprinter for secretion and suggest that different modes of secretion may exist for different Wnt proteins.

Throughout the life of a multicellular organism, individual cells of the body must communicate with each other to coordinate important processes such as growth, cell fate specification, and differentiation. Wnt proteins comprise one conserved family of secreted signaling molecules that act in various contexts, playing key roles in both embryonic development and adult homeostasis. Misregulation of Wnt signaling has been implicated in developmental abnormalities and cancer (1). Several Wnt family members have been shown to act as morphogens, signaling in a concentration-dependent manner to elicit a range of graded responses at different distances from the source of the signal (2–4). Given the importance of correctly regulating the range of Wnt signaling, many studies have been directed toward understanding how Wnt proteins are produced, secreted, and distributed within a tissue (5).

One system that has yielded insight into these questions is the fruit fly. Genetic screens in *Drosophila* uncovered the first Wnt pathway member found to play a role in Wnt-producing cells (6). This gene, *porcupine*, encodes a multipass transmembrane protein localized to the endoplasmic reticulum (ER)[3] (7). In its absence, *Drosophila* Wnt1/Wingless (Wg) protein is not secreted properly and becomes trapped in Wg-expressing cells (8). Subsequent studies showed that Porcupine is also required for the secretion of Wnt family members in different species, including mouse Wnt3a (9) and *Drosophila* Wnt5 (10, 11). More recently, another Wnt secretion component called Wntless (Wls, also known as Evenness Interrupted (Evi) and Sprinter) was identified, supporting the idea that an important layer of signal regulation takes place in Wnt-producing cells (12–14). Wls is proposed to assist in the trafficking of Wnt proteins between the trans-Golgi network and plasma membrane and, like Porcupine, is required for the secretion of various Wnt family members (15–19). While the mechanism of Wls function remains unknown, there is evidence that Porcupine is involved in the post-translational modification of Wnt proteins. Porcupine displays sequence homology to a large family of *O*-acyltransferase enzymes (20) and appears to be directly or indirectly involved in the covalent attachment of fatty acids onto Wnt proteins in the ER (9, 21).

Lipid modification has been observed on multiple Wnt family members. Specifically, several Wnt proteins are modified by the addition of palmitic acid on the most N-terminal conserved cysteine residue and palmitoleic acid on a highly conserved serine residue (9, 22–24). These sites correspond to cysteine 77 (Cys-77) and serine 209 (Ser-209), respectively, in mouse Wnt3a. Lipid modification has been shown in different instances to regulate the biological activity of signaling proteins by affecting structural stability, membrane targeting, and protein-protein interactions (25, 26). In the case of Wnt proteins, several lines of evidence have suggested that lipid modification is required for proper secretion. First, loss of Porcupine leads to loss of lipid modification and secretion defects in Wnt3a and Wingless (8, 9, 21). Second, mutating serine 209 on mouse Wnt3a resulted in a loss of Wnt3a secretion (9). Because lipid modification of *Drosophila* Wnt1/Wingless is reported to target the protein to intracellular lipid rafts (21), an interesting hypothesis is that lipid modification might play a role in secretion by directing the intracellular trafficking of Wnt proteins through specific membrane subdomains into particular organelles along the secretory pathway. One difficulty in these studies, however, lies in determining whether the observed

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[3] The abbreviations used are: ER, endoplasmic reticulum; HRP, horseradish peroxidase; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate.

secretory defects result purely from the lack of the fatty acid attachment, or whether they result from protein misfolding due to mutating or uncovering previously modified residues.

WntD (Wnt inhibitor of Dorsal) is a recently characterized Wnt family member that plays a role in dorsal-ventral patterning in the *Drosophila* embryo and also functions to regulate Toll/Dorsal-mediated antimicrobial production during the innate immune response in adult flies (27, 28). In this study, we examined regulation of WntD at the level of Wnt-producing cells. Interestingly, we found that Porcupine is not required for WntD secretion or signaling activity. Because Porcupine is involved in the lipid modification of Wnt proteins, we used a novel method for detecting protein lipidation and mass spectrometry to ask whether WntD undergoes lipid modification and found that it does not, demonstrating for the first time that lipid modification is not a universal feature of Wnt proteins. Despite its lack of lipid modification, we observed that WntD is secreted at high levels when expressed in cultured cells. We also found that WntD secretion and signaling activity are independent of the secretion factor Wntless. Thus, by studying the behavior of a naturally non-lipid-modified Wnt family member, we demonstrate that not all Wnt proteins require lipid modification, Porcupine, or Wntless for secretion and raise the interesting possibility that lipid modification may regulate the mode of secretion for different Wnt family members.

EXPERIMENTAL PROCEDURES

Cell Culture, Transfection, and Secretion Assays—Drosophila S2 cells were cultured at 25 °C in Schneider's medium supplemented with 10% fetal bovine serum (Sigma), penicillin, and streptomycin. Cells transfected with *pTub-FLAG-wg* or *pTub-FLAG-wntD* were selected using 125 μg/ml hygromycin (Sigma) to obtain stable Wnt-expressing cell lines. Mouse L cells were cultured at 37 °C with 5% CO_2 in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Sigma), penicillin, and streptomycin. Cells transfected with *pEF1a-FLAG-wnt3a* or *pEF1a-FLAG-wntD* were selected using 1 mg/ml neomycin to obtain stable Wnt-expressing cell lines. Single cell clones were screened for expression by Western blot. To assay secretion levels, 1×10^6 cells were plated for 72 h (for S2 cell lines) or 40 h (for L cell lines) before conditioned medium was collected, centrifuged at 1000 rpm for 5 min to remove non-adherent cells, and assayed for secreted protein levels by Western blot analysis using mouse M1 anti-FLAG antibody (Sigma, 1:1000) followed by HRP-conjugated anti-mouse secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA 1:20,000) and enhanced chemiluminescence (ECL) detection (PerkinElmer Life Sciences). 0.1% Ponceau S (Sigma) (w/v) in 5% acetic acid (Sigma) was used for reversible detection of total protein levels on the membrane before immunoblotting.

Metabolic Labeling of Wnt Proteins with an Azido-palmitic Acid Analog—Azido-palmitic acid analog (N_3-15) and phosphine-biotin reagents were synthesized as previously described (29). 100 μM azido-palmitic acid analog was added to complete Schneider's medium and allowed to pre-complex for 15 min at room temperature, and then added to plain S2 cells or S2 cells stably expressing FLAG-Wg or FLAG-WntD. Cells were grown in fatty acid medium for 72–86 h at 25 °C before conditioned medium was collected and centrifuged at 1000 rpm for 5 min. Secreted FLAG-tagged Wnt proteins were immunoprecipitated from the conditioned medium with anti-FLAG M1-agarose beads in the presence of 1 mM $CaCl_2$ by rotating for 3 h at room temperature or overnight at 4 °C. Beads were washed with cold 50 mM Tris, pH 7.4, 5 mM $MgCl_2$, 0.5% Nonidet P-40, 1 mM $CaCl_2$ three times and then incubated with 200 μM phosphine-biotin for 2 h at 37 °C. A ligation modeled after the Staudinger reaction forms an amide bond by coupling azide and triaryl-phosphine-biotin. Reactions with phosphine-biotin were carried out in the presence or absence of 5 mM dithiothreitol, which can prevent nonspecific oxidation of phosphine-biotin to reduce background labeling. We observed that under the described conditions, the labeling of Wnt proteins with the azido-palmitic acid analog was not affected by 5 mM dithiothreitol. Reactions were stopped by the addition of 1 ml of ice-cold acetone, incubated at −20 °C overnight, and centrifuged at 20,000 × *g* for 10 min at 4 °C. After air-drying, the beads were resuspended in 1× non-reducing SDS loading dye and analyzed by SDS-PAGE. Proteins were transferred onto nitrocellulose membranes and biotin detection carried out using streptavidin-HRP (Jackson ImmunoResearch, West Grove, PA) at 1:10,000 for 30 min at room temperature. Membranes were stripped and reprobed with anti-FLAG M2-HRP (Sigma) at 1:2000 for 1 h at room temperature to detect Wnt protein levels. In both cases, HRP-conjugated antibodies were detected with ECL.

WntD Purification, N-terminal Sequencing, and Mass Spectrometry—6 liters of conditioned medium from stable FLAG-WntD-expressing S2 cells was adjusted to 1% Triton X-100 and applied to a Cibacron blue 3GA-Sepharose column (Amersham Biosciences) equilibrated in 150 mM KCl, 20 mM Tris, 1% CHAPS, pH 7.5 as previously described (24). Fractions were eluted with 1.5 M KCl, 20 mM Tris, 1% CHAPS, pH 7.5 and analyzed by Western blot with anti-WntD antibody (28) (1:10,000) and Coomassie Blue staining. A relatively clean fraction of WntD protein was further affinity purified by binding to M1-agarose beads (Sigma) and eluting with M1 peptide (Sigma) into 0.5 M NaCl, 20 mM Tris-HCl pH 7.5, 5 mM $CaCl_2$ with or without 1% CHAPS. Purified protein was separated on SDS-PAGE and transferred to a polyvinylidene difluoride membrane before being subjected to N-terminal sequencing by Edman degradation chemistry (Protein and Nucleic Acid Facility (PAN), Stanford, CA). For mass spectrometry analysis, purified WntD protein was dialyzed into 0.5 M NaCl, 20 mM Tris-HCl, pH 7.5 and analyzed by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) on a Perseptive Voyager-DE RP mass spectrometer using internal mass standards (PAN, Stanford, CA).

Drosophila Genetics and Immunostaining—Germline clones were obtained from animals of the following genotypes: 1) *y,w,hsp70-flp;FRT80 wls^2/FRT80 P[ovoD]* x *FRT80 wls^2/TM3, hb-lacZ*, 2) *w,ovo^{svb},FRT9-2 por^{PB16}/v, FRT9-2 P[ovoD]* x *FM7, ftz-lacC/y*, and 3) *w, ovo^{svb},FRT9-2 por^{PB16}/v, FRT9-2 P[ovoD]; UAS-WntD/nanos-GAL4:VP16* x *FM7, ftz-lacC/y*.

Clones were induced by heat shocking second or third instar larvae several times at 37 °C for 2 h each at 24-h intervals.

Immunostaining was performed using standard techniques with mouse anti-Dorsal 7A4 (DSHB, Iowa City, IA) at 1:10 and rabbit anti-lacZ (Cappel, Aurora, OH) at 1:2000 using Alexa488 and Cy3 fluorescent secondaries at 1:500 each, then imaged on a Zeiss LSM5 Pascal confocal microscope at 63× magnification. Cuticle preparations were also done according to standard protocols.

Wing imaginal discs were dissected from third instar larvae of the following genotypes: 1) *y, w, f, por^{2E}/y* or +; *UAS-wg* or *UAS-wntD*/+; *dpp-GAL4*/+, 2) *wg-GAL4/UAS-wntD* or *UAS-wg*; *wls^{1}/wls^{1}* or +, and 3) *UAS-wntD* or *UAS-wg*/+; *dpp-GAL4/UAS-rab1^{S25N}*.

Immunostaining was done according to standard techniques with mouse anti-Wingless 4D4 (DSHB) at 1:50 or rabbit anti-WntD at 1:10,000 and Alexa488 or Cy3 fluorescent secondary antibodies at 1:500, then imaged on a Zeiss Axioplan2 fluorescence microscope equipped with an Axiocam MRm camera at 63× magnification. All images were processed with Adobe Photoshop, and figures were prepared using Adobe Illustrator.

RESULTS

Secretion and Function of WntD Do Not Require Porcupine—To better understand WntD signal production, we asked whether Porcupine was necessary for WntD secretion. To address these requirements, we turned to the *Drosophila* wing imaginal disc where protein secretion can be visualized by immunostaining. It has been previously observed that in the absence of Porcupine or Wntless, a drastic shift can be seen in the distribution of Wingless protein (12, 13, 30). In wild-type larval wing discs, Wingless is diffusely centered around the stripe of cells at the dorsal-ventral boundary of the disc where it is expressed and in punctate structures in and around the expressing cells. In the wing discs of *porcupine* or *wntless* mutant larvae however, Wingless staining is restricted to the cells that express *wingless*. Wingless staining is also more intense in these cells as compared with wild-type wing discs, likely as a result of Wingless accumulation in these cells because of lack of secretion. This alteration in protein distribution does not appear to be due to changes in *wingless* transcription, as the expression of a *wg-lacZ* transgene is unaffected by the absence of *wntless* in the larval wing disc (12). Similarly, we observed no changes in the intensity of *wg-lacZ* expression in the absence of *porcupine* in the larval wing disc when compared with wild-type wing discs (data not shown).

To test whether WntD secretion requires Porcupine, we used *decapentaplegic-GAL4* (*dpp-GAL4*) to express *UAS-wntD* or a *UAS-wingless* control in male larvae zygotically mutant for *porcupine* or in heterozygous control larvae, which are viable and have no *wingless* phenotype. The *dpp* promoter drives expression in a stripe of cells along the anterior-posterior boundary of the larval wing imaginal disc. We examined the pattern of protein distribution in wing discs by immunostaining against WntD or Wingless. Heterozygous control and hemizygous mutant wing discs were imaged under the same conditions. As expected, in control wing discs, immunostaining revealed a diffuse pattern of protein distribution centered along a stripe at the anterior-posterior boundary for both WntD and Wingless (Fig. 1, *A* and *C*). The staining pattern was characterized by the appearance of WntD or Wingless protein in punctate structures in the expressing cells as well as in cells surrounding the region of *dpp-GAL4*-driven expression. (Fig. 1, *A* and *C*, *arrows*). However, in *porcupine* hemizygous mutant wing discs, where Wingless secretion is known to be impaired, Wingless protein was only detected in a clear stripe of cells in the *dpp-GAL4*-driven expression domain and was not observed in characteristic punctate structures (Fig. 1*B*). Wingless staining was also of greater intensity in these cells compared with in control wing discs. We interpret this shift in the distribution of Wingless protein as an indication that it is being retained in cells and not secreted. In contrast, we found that WntD protein distribution was unaffected by loss of Porcupine. Similar to what we observed in control wing discs, WntD was detected in a diffuse gradient characterized by punctate structures in *porcupine* hemizygous mutant wing discs (Fig. 1*D*), suggesting that the secretion of WntD protein does not require Porcupine.

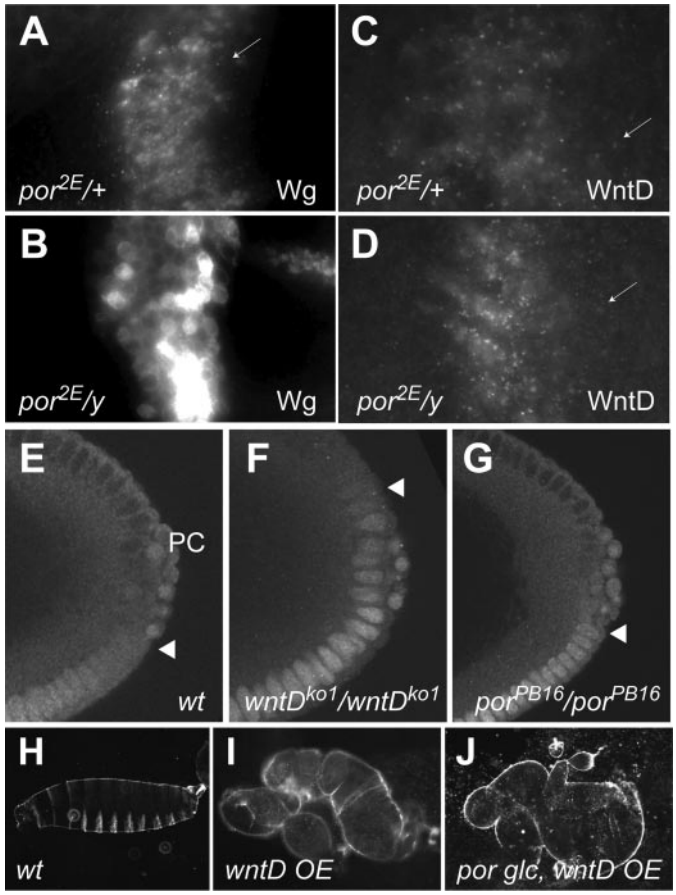

FIGURE 1. **WntD secretion and signaling activity do not require Porcupine.** *A–D*, third instar larval wing discs from heterozygous (*A* and *C*) or *porcupine* hemizygous zygotic mutant (*B* and *D*) animals in which *UAS-wingless* (*A* and *B*) or *UAS-wntD* (*C* and *D*) was expressed using *dpp-GAL4*. Wing discs were dissected and stained using antibodies against Wingless (Wg) or WntD protein and are displayed at 63× magnification with the dorsal-ventral axis oriented vertically and the anterior-posterior axis oriented horizontally. *E–G*, sagittal view of the posterior pole region of blastoderm stage embryos stained using antibodies against Dorsal to visualize the nuclear accumulation of Dorsal protein (ventral side, *down*; dorsal side, *up*). *Arrowheads* indicate the dorsal-most level of Dorsal nuclear accumulation in somatic cells, shown relative to the pole cells (*PC*) in wild type (*E*), *wntD* mutant (*F*), or *porcupine* maternal and zygotic mutant (*G*) embryos. *H–J*, cuticle preparations from wild-type embryos (*H*) or embryos in which *UAS-wntD* was overexpressed (*OE*) in the maternal germline using *nanos-GAL4:VP16* in a wild type (*I*) or *porcupine* germline clone (*por glc*) mutant (*J*) background.

To examine whether the signaling activity of WntD is dependent on Porcupine, we generated embryos that were maternally and zygotically mutant for *porcupine* to see whether they exhibited defects in WntD signaling. WntD functions as an inhibitor of Toll/Dorsal signaling by preventing the nuclear accumulation of Dorsal (27, 28). During *Drosophila* development, WntD is expressed at the anterior and posterior poles of the blastoderm stage embryo where it restricts the range of Toll/Dorsal signaling in somatic cells during the establishment of dorsal-ventral polarity. Consequently, *wntD* mutants show an expansion of nuclear Dorsal into the pole regions of the embryo. In contrast, the nuclear accumulation of Dorsal in somatic cells of wild-type embryos stops at or near the level of the pole cells (also called germline precursor cells), although Dorsal protein can also be observed in the pole cells themselves (31). We compared the localization of nuclear Dorsal in somatic cells at the posterior pole of *porcupine* mutant embryos (Fig. 1*G*) to wild type (Fig. 1*E*) and *wntD* (Fig. 1*F*) mutant embryos. We found that *porcupine* mutant embryos did not phenocopy *wntD* mutant embryos, suggesting that WntD signaling does not require Porcupine.

We next overexpressed WntD in the absence of Porcupine. Overexpression of WntD in the female germline leads to high levels of WntD protein in blastoderm stage embryos, inhibiting the nuclear accumulation of Dorsal and subsequent ventral cell fate specification. This leads to embryos that produce only dorsal cuticle (28) (Fig. 1*I*) as opposed to wild-type embryos that produce both dorsal and ventral cuticle (Fig. 1*H*). We overexpressed WntD in the germline of *porcupine* germline clone females using *nanos-GAL4:VP16* and examined the embryonic cuticles of the resulting progeny. We found that all progeny, even those maternally and zygotically mutant for *porcupine*, displayed dorsalized embryonic cuticles as a result of WntD overexpression (Fig. 1*J*), confirming our previous conclusion that WntD signaling does not require Porcupine. Because Porcupine is involved in post-translational lipid modification of Wnt proteins, we next asked whether WntD might represent a non-lipid-modified member of the Wnt family.

WntD Does Not Undergo Lipid Modification—To determine whether WntD undergoes lipid modification, metabolic labeling studies were performed with azide-modified fatty acids that function as chemical reporters of fatty acylation on proteins (29). *Drosophila* S2 cells stably expressing Wnt proteins tagged with an N-terminal FLAG (DYKDDDDK) epitope tag were incubated with an azido-palmitic acid analog (N_3-15). Secreted Wnt proteins were immunoprecipitated from cell supernatants with antibodies directed against the FLAG tag. Metabolically incorporated azido-palmitic acid analogs were reacted with phosphine-biotin via the Staudinger ligation, allowing for the rapid and sensitive detection of lipid modification on Wnt proteins using standard biotin detection techniques. We found that while *Drosophila* Wnt1/Wingless (Wg) was specifically labeled by the azido-palmitic acid analog, no label was detected on WntD, even though levels of WntD protein are similar to levels of Wg protein (Fig. 2*A*). The same results were observed when comparing labeling of WntD with the azido-palmitic acid analog to a mouse Wnt3a control protein in stably transfected mouse L cells (data not shown).

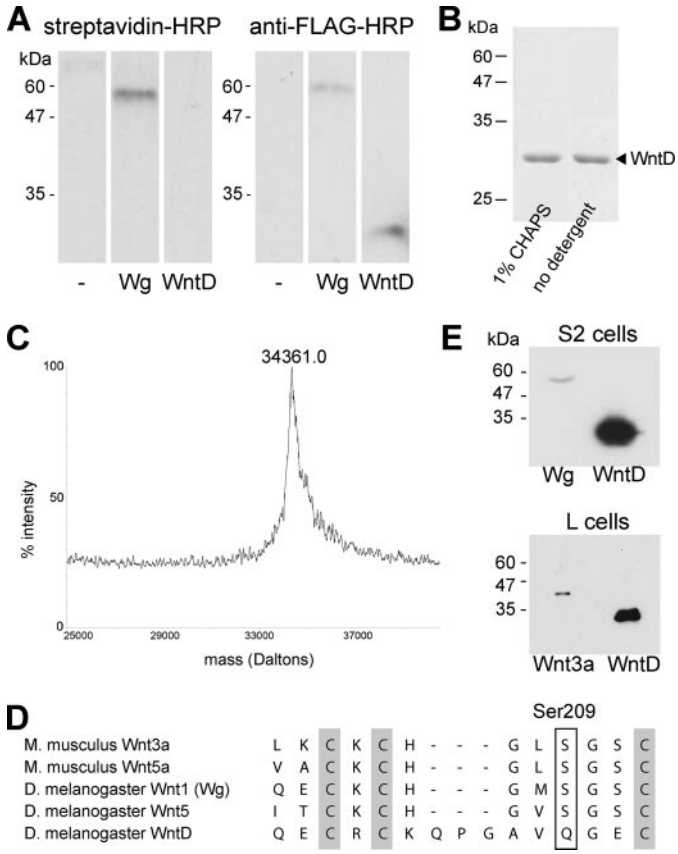

FIGURE 2. **WntD does not undergo lipid modification and is secreted at high levels.** *A*, analysis of protein lipidation. S2 cells expressing either nothing (*lane 1*), FLAG-Wingless (*lane 2*), or FLAG-WntD (*lane 3*) were incubated with an azido-palmitic acid analog and secreted Wnt proteins were pulled down from the supernatant using anti-FLAG beads before reaction with phosphine-biotin via the Staudinger ligation. The incorporated azido-palmitic acid analog was detected with streptavidin-HRP (*first panel*). Wg (52 kDa) or WntD (34 kDa) protein levels were detected by stripping and reprobing the membrane with an anti-FLAG antibody conjugated to HRP (*second panel*). *B*, Coomassie Blue-stained protein gel showing purified FLAG-WntD protein in detergent (*lane 1*) and non-detergent (*lane 2*) conditions. *C*, deconvoluted mass spectrum of intact FLAG-WntD protein. *D*, amino acid sequence alignment between several Wnt family members, including *Drosophila* WntD, of residue serine 209 from mouse Wnt3a (*boxed*) and the surrounding sequence, which contains conserved cysteine residues (*shaded*). *E*, supernatant from S2 cells stably expressing FLAG-Wg (S2 cells, *lane 1*) or FLAG-WntD (S2 cells, *lane 2*) or L cells stably expressing FLAG-Wnt3a (L cells, *lane 1*) or FLAG-WntD (L cells, *lane 2*) was collected, centrifuged, and detected by Western blot with an anti-FLAG antibody. Total protein levels in each lane were confirmed to be similar by reversible Ponceau S staining of the membrane before anti-FLAG detection (data not shown).

To confirm the absence of lipid modification, secreted FLAG-tagged WntD protein was purified from medium conditioned by stably transfected S2 cells and analyzed by mass spectrometry. In a two-step purification process, high salt elution fractions from affinity chromatography on a blue-Sepharose column were subjected to FLAG tag affinity purification, resulting in a pure fraction of WntD protein as assessed by Coomassie Blue staining (Fig. 2*B*, *lane 1*). Purification of other Wnt family members has been carried out in the presence of the detergent CHAPS, as this is important for the maintenance of their solubility and stability (24). However, we found that purified WntD protein is stable in the absence of detergent (Fig. 2*B*, *lane 2*), consistent with the notion that it lacks hydrophobic lipid modification. The total intact mass of purified WntD protein was

then measured by matrix-assisted laser desorption/ionization (MALDI) mass spectrometry. N-terminal protein sequencing of the first twenty amino acid residues of purified FLAG-WntD was performed and confirmed that the signal sequence of the protein was cleaved at the expected site (data not shown). Therefore, the predicted total mass of FLAG-WntD protein in the absence of any modification is ~34,353 daltons. Note that the predicted mass reflects the addition of four residues (LAAA) linking the FLAG tag to WntD. The covalent addition of palmitate or palmitoleic acid would be reflected by the addition of 238 or 236 daltons, respectively, to the total observed mass. In two separate experiments, the total mass of purified FLAG-WntD protein was measured to be 34,357 daltons and 34,361 daltons (second experiment shown in Fig. 2*C*). Given an anticipated mass accuracy of 0.1% of the molecular weight of the protein (~34 daltons in this case), total intact mass measurements of purified WntD protein do not indicate the presence of lipid modification.

In view of these results, we examined the amino acid sequence of WntD at the two sites of lipid modification found on other Wnt family members. Interestingly, we found that although the N-terminal cysteine residue corresponding to Cys-77 on mouse Wnt3a is conserved, the serine residue corresponding to Ser-209, which is highly conserved in other Wnt proteins, is not conserved in WntD (Fig. 2*D*). Ser-209 is modified by the addition of palmitoleic acid on Wnt3a (9). Takada *et al.* (9) showed that mutating Ser-209 leads to a complete loss of lipid modification even though Cys-77 is still present. We recognize that our laboratory has previously reported that *Drosophila* Wnt8 (WntD was formerly designated DWnt8) was modified by the attachment of palmitic acid on Cys-51, the residue corresponding to Wnt3a Cys-77 (24). However, in light of our reexamination of this issue using a sensitive new labeling method and total intact mass measurements, we now believe that WntD does not undergo lipid modification. One possibility that is consistent with this and with the data from Takada *et al.* is that lack of lipid modification on the second site (corresponding to Ser-209) leads to a complete loss of fatty acylation on both potential sites of lipid modification. Thus WntD, which naturally lacks the second lipid modification site, would not undergo lipid modification at the first site.

WntD Is Secreted at High Levels in Cell Culture—We observed that in cultured cells WntD was consistently secreted and released from the cell surface into the cell supernatant at very high levels relative to other Wnt proteins, including *Drosophila* Wingless and mouse Wnt3a (Fig. 2*E*). Total protein levels in the supernatant were equivalent as assessed by Ponceau S staining (data not shown). This observation was true for stably transfected *Drosophila* S2 cell lines that expressed Wnt proteins and for multiple different clonally selected lines of stably transfected mammalian L cells, as well as for transient transfections.

We also recalled that when we expressed *UAS-wntD or UAS-wg* with *dpp-GAL4* in larval wing discs, WntD protein distribution appeared to be more diffuse than Wingless protein distribution around the stripe of expression (Fig. 1*A* compared with Fig. 1*C*), perhaps consistent with WntD being secreted or released from expressing cells more efficiently than Wingless.

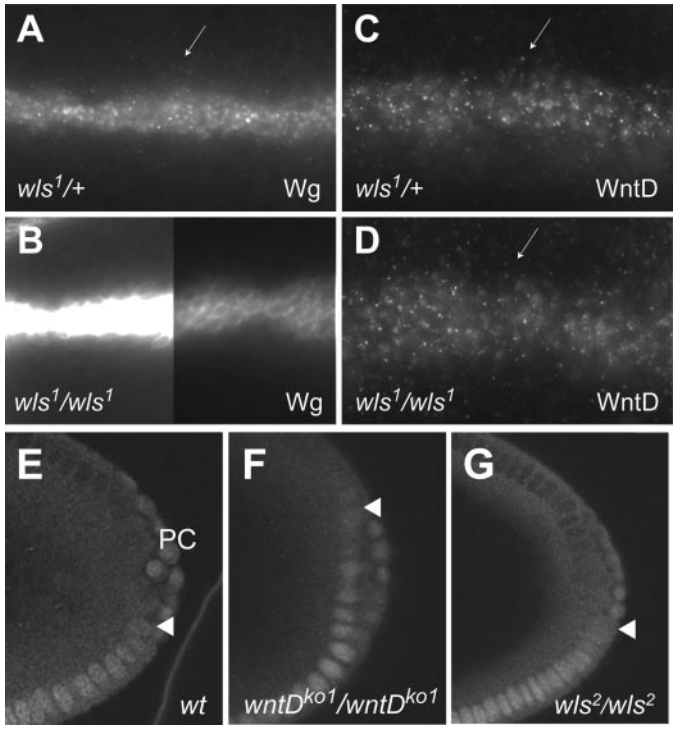

FIGURE 3. **WntD secretion and signaling activity do not require Wntless.** *A–D*, third instar larval wing discs from heterozygous (*A* and *C*) or *wntless* homozygous zygotic mutant (*B* and *D*) animals in which *UAS-wingless* (*A* and *B*) or *UAS-wntD* (*C* and *D*) was expressed using *wingless-GAL4*. Wing discs were dissected and stained using antibodies against Wingless (Wg) or WntD protein and are displayed at 63× magnification with the dorsal-ventral axis oriented vertically and the anterior-posterior axis oriented horizontally. The intense staining pattern of Wingless protein in *wntless* homozygous mutants is shown imaged under conditions identical to those used for heterozygous wing discs (*left half of panel B*) and at a lower exposure (*right half of panel B*)). *E–G*, sagittal view of the posterior pole region of blastoderm stage embryos stained using antibodies against Dorsal to visualize the nuclear accumulation of Dorsal protein (ventral side, *down*; dorsal side, *up*). *Arrowheads* indicate the dorsal-most level of Dorsal nuclear accumulation in somatic cells, shown relative to the pole cells (*PC*) in wild type (*E*), *wntD* mutant (*F*), or *wntless* maternal and zygotic mutant (*G*) embryos.

Secretion and Function of WntD Do Not Require Wntless—Given that WntD functions efficiently in the absence of Porcupine, we next asked whether WntD might also be independent of other components of the typical Wnt secretory pathway. To examine the requirements of WntD secretion and signaling activity for Wntless, we used similar approaches as those described for Porcupine. *wingless-GAL4* (*wg-GAL4*) was used to express *UAS-wntD* or a *UAS-wingless* control in homozygous *wntless* mutant larvae or in heterozygous control larvae, which are viable and have no *wingless* phenotype. The *wingless* promoter drives expression in a stripe of cells along the dorsal-ventral boundary of the wing imaginal disc. We immunostained using Wingless or WntD antibodies and imaged heterozygous control and homozygous mutant wing discs under the same conditions. As seen before, in control wing discs, immunostaining revealed a diffuse pattern of Wingless or WntD protein distribution that was centered around the stripe of cells, where they were expressed and characterized by the appearance of protein in punctate structures in both the expressing cells and cells more distant to the source (Fig. 3, *A* and *C*), indicating that the proteins were secreted. In *wntless* homozygous mutant wing discs, Wingless protein was detected at intense levels only

in cells along the dorsal-ventral boundary where *wingless* is expressed and punctuate staining was not observed (Fig. 3*B*, shown imaged under conditions identical to those used for control wing discs (*left half of panel*) and at a lower exposure (*right half of panel*)). This shift in protein distribution indicated that Wingless was not secreted in the absence of Wntless. However, WntD protein was still observed outside of WntD-expressing cells, both diffusely and in punctate structures, in *wntless* homozygous mutant wing discs (Fig. 3*D*), suggesting that the secretion of WntD does not require Wntless.

To test whether the signaling activity of WntD is dependent on Wntless, we generated embryos that were maternally and zygotically mutant for *wntless* to see whether they exhibited defects in WntD signaling. As in the previous set of experiments with Porcupine, we assayed WntD signaling by looking at the expansion of nuclear Dorsal in somatic cells at the posterior pole region of blastoderm stage embryos. We compared *wntless* mutant embryos (Fig. 3*G*) to wild type (Fig. 3*E*) and *wntD* mutant embryos (Fig. 3*F*), and found that *wntless* mutants did not exhibit an expansion of nuclear Dorsal in somatic cells at the posterior pole, suggesting that WntD signaling is intact and that it does not require Wntless.

Secretion of WntD Requires the Early Secretory Pathway Component Rab1—Rab1 GTPase is required for trafficking vesicles carrying newly folded proteins between the ER and the Golgi apparatus. We co-expressed a dominant negative form of Rab1, *UAS-rab1^{S25N}* (32), together with *UAS-wntD* using *dpp-GAL4* to examine how loss of Rab1 function would affect WntD secretion. We found that WntD protein was more concentrated in the producing cells when expressed together with a dominant negative version of Rab1 (Fig. 4*B*) than when expressed alone (Fig. 4*A*). Because of the intensity of WntD protein staining, the wing disc in Fig. 4*B* is shown imaged with a 10-fold lower exposure time than the wing disc in Fig. 4*A*. This demonstrates that WntD secretion, as assessed by *in vivo* imaging of protein distribution, can be disrupted by inhibition of an early and general secretory pathway component.

The function of Rab1 in the secretion of other Wnt proteins has not been previously examined. To address this issue, we co-expressed dominant negative Rab1 together with *UAS-wingless* using *dpp-GAL4*. Although the overall morphologies of the resulting wing discs were normal, we found that the pattern of *dpp*-driven Wingless expression was unexpectedly broadened when *UAS-rab1^{S25N}* was co-expressed (Fig. 4*D*). Even so, Wingless secretion appeared to be impaired by loss of Rab1 function (Fig. 4, *C* and *D*). Wingless staining intensity was not as greatly affected by the presence of dominant negative Rab1 as observed for WntD (note that the images in Fig. 4, *C* and *D* were taken with the same exposure times). It is possible that although Rab1 is involved in Wingless secretion, it is not as strictly required as it is for WntD secretion. An alternative explanation is that greater amounts of WntD protein accumulate in producing cells in the absence of secretion because WntD is normally produced and secreted at higher levels than Wingless.

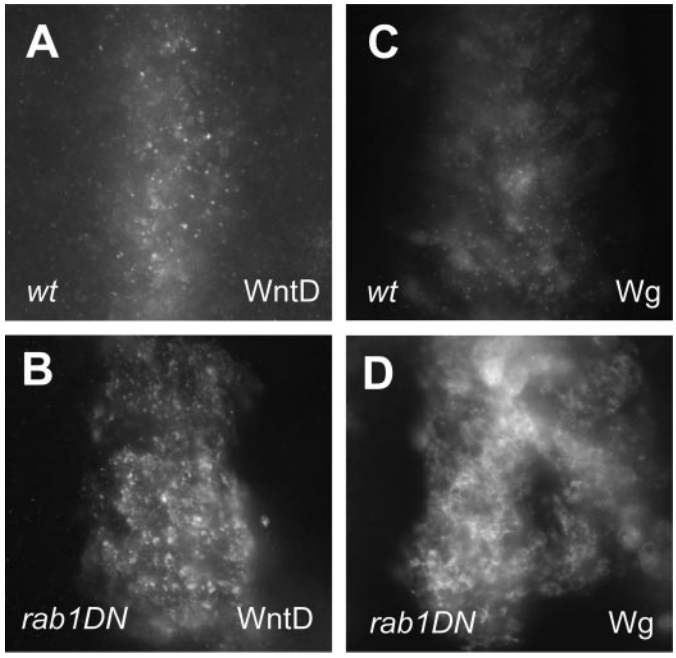

FIGURE 4. **WntD secretion requires the early secretory pathway component Rab1.** Third instar larval wing discs in which *UAS-wntD* or *UAS-wingless* alone (*A* and *C*) or *UAS-wntD* or *UAS-wingless* together with dominant negative *UAS-rab1^{S25N}* (*B* and *D*) were expressed using *dpp-GAL4*. Wing discs were dissected and stained using antibodies against WntD or Wingless (Wg) protein and are displayed at 63× magnification with the dorsal-ventral axis oriented vertically and the anterior-posterior axis oriented horizontally. WntD staining in *panel B* is shown imaged at a 10-fold lower exposure time than *panel A* to show non-saturating conditions. Wingless staining in *panels C* and *D* are shown imaged under identical conditions.

DISCUSSION

The regulation of secreted signaling proteins by post-translational lipid modification has become a topic of great interest in recent years. For example, both Hedgehog and the EGFR ligand Spitz were found to be lipid-modified, and these modifications play an important role in regulating their signaling activities (33, 34). Many Wnt family members are also lipid-modified, and it has been hypothesized that lipid modification of Wnt proteins is required for their secretion. In addition, lipid modification of Wnt proteins may play a role in their distribution through tissues, as overexpression of *porcupine* in the chick neural tube leads to a more restricted range of Wnt1 and Wnt3a signaling activity (35). Although lipid modification is generally presumed to be a universal feature of Wnt proteins, the results of this study bring to light the presence of a non-lipid-modified Wnt family member. This presented us with the opportunity to study the behavior of a naturally non-lipid-modified Wnt protein in its wild-type form.

We find that WntD appears to be secreted in a manner different from other Wnt proteins. For example, WntD is secreted at extremely high levels in cell culture, and its secretion and function are independent of Porcupine and Wntless *in vivo*. WntD secretion does, however, maintain a requirement for Rab1, an early component of the secretory pathway that regulates the transport of vesicles from the ER to the cis-Golgi compartment. While Porcupine is involved in lipid modification in the ER, Wntless is thought to escort proteins such as Wingless between the trans-Golgi network and the plasma membrane.

These results suggest that WntD might be sorted to an alternative secretory route, possibly at the level of the trans-Golgi network, a major sorting site for intracellular trafficking in the secretory pathway.

These observations raise interesting questions about the role of post-translational lipid modification in regulating Wnt secretion. It has been suggested that lipid attachments may function to target modified Wnt proteins such as Wingless to specific intracellular membrane subdomains that direct the trafficking of the protein through the secretory pathway along a particular route that could, for example, lead to packaging of the protein into secretory vesicles after exit from the trans-Golgi network. Perhaps WntD is targeted to a different membrane subdomain due to its lack of lipid modification. Consequently it is sorted to an alternative secretory route, independent of carrier proteins such as Wntless, leading to robust levels of secretion. It is interesting to consider the possible biological role of an alternative mode of secretion for WntD. WntD is known to act in the adult *Drosophila* innate immune response by inhibiting the Toll/Dorsal-mediated antimicrobial response. It is possible that this unique function requires that it be distributed and act systemically in rapid response to infection, aided in part by robust levels of secretion and a greater range of action for this non-lipid-modified Wnt protein.

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