Secreted Wingless-interacting molecule (Swim) promotes long-range signaling by maintaining Wingless solubility

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Lipid-modified Wnt/Wingless (Wg) proteins can signal to their target cells in a short- or long-range manner. How these hydrophobic proteins travel through the extracellular environment remains an outstanding question. Here, we report on a Wg binding protein, Secreted Wg-interacting molecule (Swim), that facilitates Wg diffusion through the extracellular matrix. Swim, a putative member of the Lipocalin family of extracellular transport proteins, binds to Wg with nanomolar affinity in a lipid-dependent manner. In quantitative signaling assays, Swim is sufficient to maintain the solubility and activity of purified Wg. In Drosophila, Swim RNAi phenotypes resemble Wg loss-of-function phenotypes in long-range signaling. We propose that Swim is a cofactor that promotes long-range Wg signaling in vivo by maintaining the solubility of Wg.

Wnt proteins comprise a conserved family of secreted signaling molecules with key functions during embryonic development and adult homeostasis (1). Wnt signaling is initiated by the binding of Wnt to its receptors, Frizzled (Fz) and Arrow/LRP5/6 (2, 3), which leads to the stabilization and subsequent translocation of β-catenin to the nucleus where it associates with members of the Tcf/Lef family of transcription factors to regulate target genes (4, 5). Aberrant Wnt signaling contributes to a variety of developmental abnormalities and adult diseases (6); therefore, elucidating the mechanisms of Wnt signal transduction is an area of intense research.

Wnt proteins can act as morphogens (7–9), secreted molecules that affect tissue organization by providing spatial information in the form of a concentration gradient. In the developing Drosophila wing imaginal disc, Wingless (Wg) is secreted by a narrow stripe of cells at the dorsoventral (DV) boundary. It then diffuses through a field of cells to activate transcription of high-threshold target genes such as senseless (sens) close to the Wg-producing cells and low-threshold genes such as distalless (dll) farther away (10–12). This finding implies that Wg travels away from its site of production through the extracellular environment while maintaining an active conformation.

Wnt proteins are dually lipid modified by the covalent attachment of palmitic acid (9, 13, 14) and palmitoleic acid (15), causing them to be hydrophobic (14), and Wnts may be membrane-associated under many circumstances (16, 17). This finding presents a paradox: how is it possible that Wnts affect cells at a distance if their lipid moieties confer high affinity for cell membranes? Experiments in Drosophila show that a membrane-tethered form of Wg is sufficient to function as a short-range inducer as it can rescue wg-null embryos and induce transcription of short- but not long-range target genes in larval wing discs (12). This absence of transcriptional activation of long-range Wg target genes indicates that diffusion away from the site of production is necessary for proper Wg signaling. Extracellular Wg has been detected in membranous lipoprotein particles (18), which may contribute to Wg transport; however, Wg signaling activity of these particles has not been reported. Another possibility is that Wnts diffuse freely from membranes, but this possibility raises the question of how a hydrophobic protein is able to travel through the aqueous extracellular environment without aggregating.

In this paper, we describe an extracellular Wg binding protein called Secreted Wg-interacting molecule (Swim), a putative member of the Lipocalin family of transport proteins. We show that Swim binds to Wg with nanomolar affinity in a palmitate-dependent manner and maintains Wg solubility and signaling activity. Reduction of swim expression in vivo shortens the distribution of extracellular Wg and leads to impaired long-range Wg signaling activity. These data suggest a unique carrier function of Swim to mediate the morphogenetic activity of the hydrophobic Wg protein.

Results

Swim Is Necessary for the Maintenance of Wg Signaling Activity in Vitro. Evidence for the existence of a cofactor for Wg signaling activity arose during the purification of Wg from a Schneider2 (S2) cell line overexpressing Wg. On purification of Wg (Fig. L4), we observed a loss of Wg-dependent signaling activity (Fig. 1B) using Wg reporter cells (an S2 cell line stably transfected with β-gal, SuperTOPFlash, and LacZ) (19, 20). This finding suggested that a necessary endogenous cofactor secreted by S2 cells was lost during the purification process. Consistent with this hypothesis, we found that the signaling activity of purified Wg was restored in medium conditioned by S2 cells (S2CM) (Fig. 1B).

We reasoned that putative Wg-interacting factors could be present as minor fractions in our purified Wg preparations. On examination of mass spectrometry data generated from purified Wg, we detected peptides of a protein encoded by CG3074. Interestingly, preparations of mammalian Wnt3a and Wnt5a purified from mouse L cells also contained peptides of the mammalian homolog of CG3074, Lipocalin7 (Lcn7) (21). The Drosophila CG3074 locus encodes a 431-aa protein (Fig. 1C) that has a Sulfotransferase ECM interactions, a Lipocalin Signature Motif (amino acids 252–265), which is common to Lipocalin family members, and a Cathepsin B protease domain (amino acids 186–409), although the lack of a conserved residue in the active site likely renders it catalytically inactive (21). The amino acid sequence also includes a putative N-linked glycosylation site and an N-terminal signal peptide, consistent with this protein being secreted.


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analysis revealed orthologs in species ranging from worms to humans (Fig. S1).

Because Lipocalins facilitate the extracellular transport of hydrophobic proteins (22, 23), we hypothesized that CG3074 could be a potential binding partner of Wg. We overexpressed a tagged form of CG3074, which we refer to hereafter as Swim, to alanine (FWgC93A). To compare signaling activities of WT and mutant Wg, we used a coculture assay in which we incubated Wg reporter cells with either FWg- or FWgC93A-secreting cells. In this paracrine assay, FWgC93A signaled to neighboring cells, although the level of activity was reduced 10-fold compared with FWg (Fig. 3A). The decrease in activity was not caused by lower levels of FWgC93A protein, because comparable amounts of FWg and FWgC93A were produced (Fig. S4).

To compare the activities of FWg and FWgC93A in solution, media conditioned by WT S2 or FWg- or FWgC93A-producing cells were added to Wg reporter cells. Although the FWg-conditioned medium exhibited robust Wg signaling activity, no activity could be detected with the FWgC93A CM (Fig. 3B); however, the overall level of FWgC93A protein was higher than the level of FWg (Fig. 3B, Inset). This finding suggests that FWgC93A quickly loses activity after being secreted into the medium. To further analyze the mutant protein, we fractionated CM of FWgC93A-producing cells using size exclusion chromatography. In contrast to WT Wg, which is eluted from the size exclusion column at an apparent molecular mass of 50–100 kDa, FWgC93A was detected in higher molecular mass fractions, close to the excluded volume of the column (>1,300 kDa) (Fig. 3C), suggesting that secreted FWgC93A readily aggregates.

We hypothesized that the insolubility of FWgC93A would explain the discrepancy between the previous experiments, where FWgC93A was able to trigger Wg signaling when expressed in cells immediately adjacent to Wg reporter cells (Fig. 3A) but not when provided in the form of CM (Fig. 3B). To test this hypothesis, we plated Wg reporter cells and Wg-secreting cells together at
dependent on the palmitate modification by binding to the signaling molecule in a manner that is necessary for Wg signaling activity in S2 cells (Fig. 1). These results, together with data from previous experiments (Fig. 2 D and E), suggest that Swim prevents Wg from aggregating in solution, thus losing its signaling activity.

The WT protein, palmitoylated at Cys-93, is more soluble in the aqueous extracellular environment despite being more hydrophobic than WgC93A. To explain this paradoxical finding, we propose that Swim maintains Wg solubility through a direct association with the Wg palmitate moiety. If true, loss of the necessary acylation site should prevent Swim-mediated activity. We performed a competition experiment using palmitic acid (free palmitate; IC50 = 1.54 nM), palmitoleic acid (free palmitoleate; IC50 does not converge), or BSA (IC50 does not converge).

**Purified Swim Is Sufficient for Wg Signaling Activity In Vitro.** Swim is necessary for Wg signaling activity in S2 cells (Fig. 1E), and purified Swim maintains Wg solubility through a high-affinity interaction (Fig. 2 C and D). We therefore reasoned that Swim would be sufficient to sustain Wg signaling activity. To investigate this possibility, we innovated Wg reporter S2 cells with purified Wg and increasing concentrations of purified SwimFL or SwimCT, as well as BSA or β-lactoglobulin as controls. We found that purified SwimFL, but not SwimCT, was sufficient to maintain Wg signaling activity in the absence of conditioned media (Fig. 4A). This effect was concentration-dependent, because increasing the amount of SwimFL ultimately led to a decrease in Wg activity. Swim exhibits a nanomolar binding affinity for Wg (similar to DFz2CRD); therefore, we hypothesized that high concentrations of Swim were outcompeting DFz2 for access to Wg. To examine this hypothesis, we immobilized DFz2CRD as a positive control, Swim, and then measured the ability of purified Wg to bind to these proteins in the presence of increasing concentrations of free Swim. Swim effectively competed the interaction between Wg and DFz2CRD (Fig. 4B), although BSA could not (Fig. S5), showing that the observed dose sensitivity of the Swim-Wg interaction is likely caused by a molecular competition event between Swim and at least one of the Wg receptors, DFz2.

We used a cell surface binding assay to visualize how increasing concentrations of Swim affect the ability of Wg to interact with its receptors and quantified Wg binding by measuring the pixel intensities of each DFz2-expressing cell and purified Wg protein bound to them. In the absence of Swim, purified Wg did not bind to the surface of DFz2-expressing cells (Fig. 4 C, C′, and C″) and exhibited a Wg:Fz ratio of 0.55 (Fig. 4D). In accordance with the in vitro activity assay (Fig. 4A), we found that Wg bound maximally to DFz2-expressing cells in the presence of 3 nM Swim (Fig. 4 E, E′, and E″) and displayed an increased Wg:Fz ratio of 1.3 (Fig. 4F). Increasing the concentration of Swim to 8 nM decreased Wg binding (Fig. 4 G, G′, and G″) and reduced the Wg:Fz ratio to below the ratio observed in the absence of Swim (Fig. 4H).
These results are consistent with a model in which Wg can exist in two different complexes: a Wg-Swim and a Wg-Receptor complex; the relative concentrations of these complexes are dependent on the dissociation constants and the concentrations of the individual components. At functional concentrations, Swim maintains the solubility of extracellular Wg, thereby promoting its ability to interact with its receptors. However, at high concentrations, although Swim still maintains Wg solubility (Fig. 2D), productive Wg-Receptor complex formation is reduced because of competition by Swim.

**In Vivo Reduction of swim Decreases the Range of Extracellular Wg Diffusion and Leads to wg Loss-of-Function Phenotypes.** To analyze the function of Swim in vivo, we first surveyed a number of *Drosophila* tissues for *swim* RNA levels (Fig. 5A) and found it to be expressed in the wing imaginal disc in a diffuse pattern, with higher levels at the DV boundary (Fig. 5B). Because the morphogen activity of Wg is well-established in the wing tissue, we chose to study Swim activity here (7, 11, 12). To examine possible phenotypes, we created two nonoverlapping Upstream Activation Sequence (UAS)-RNAi constructs (Fig. S6) (29) and acquired a *swim* RNAi line (*TRIP-swim*; referred to as *TRIP-swim*) from the *Drosophila* RNAi Screening Center (http://insitu.fruitfly.org). We expressed the *swim* RNAi constructs using the ubiquitous driver *actin-Gal4* (*act-Gal4*) and detected decreased levels of *swim* transcript in the wing imaginal disc using in situ hybridization (Fig. 5C) and quantitative RT-PCR (Fig. S7). Expression of *swim* RNAi was partially lethal (Table S1), with lethality occurring during larval to pupal but not embryonic stages (Table S2). Although Wg has essential functions during embryogenesis, many of these functions are thought to be short-range signal-dependent functions (12, 30). In situ analysis of *swim* expression indicates that *swim* is not expressed until embryonic stage 13 (http://www.fruitfly.org/cgi-bin/ex/insitu.pl), which is subsequent to critical Wg signaling events. To eliminate potential maternal contribution of Swim, we expressed *swim* RNAi in the germ line using *nanos-Gal4* (*nos-Gal4*). The cuticles of embryos with maternally expressed *swim* RNAi were indistinguishable from WT (Fig. 5E compared with Fig. S5D). Although we cannot exclude that a possible maternal Swim contribution was not completely eliminated by the *swim* RNAi-*nos-Gal4* combination, the lack of embryonic phenotypes is in concordance with the finding that Swim is not required for short-range signaling events (below).

To examine the molecular phenotypes caused by reduction of *swim* expression, we measured the ability of extracellular Wg to diffuse and signal in *swim* RNAi third instar larval wing discs. In *swim* RNAi wing discs, the range of extracellular Wg protein distribution seemed to be diminished compared with WT (Fig. 5F and G). On quantification, we found that levels of extracellular Wg were significantly lower in *swim* RNAi discs in every region of the wing pouch, except at the DV boundary where Wg is produced (Fig. 5H and I and Fig. S8). Notably, although reducing *swim* expression disrupted Wg diffusion, Wg secretion seemed to be unaffected, which was observed at the DV boundary (Fig. 5H and I) and measured in cell culture (Fig. S9).

We expressed *swim* RNAi using *engulfed-Gal4* (*en*) to see if decreasing *swim* specifically within the posterior domain of the wing pouch would lead to a discernable effect on the extracellular distribution of Wg within the posterior but not the anterior domain. Because of potential compensation from anteriorly produced Swim, we examined the difference in Wg diffusion across a section of cells distant from the anteroposterior (AP) boundary within...
These two domains (Fig. 5J). Extracellular Wg in the posterior region of the wing had a shortened range of extracellular distribution relative to Wg in the anterior region (Fig. 5J and K). To quantify the observed effect, the ratio of extracellular Wg in these posterior and anterior regions of en>swim RNAi wing discs was compared with WT discs and found to be significantly lower in regions farther from the DV boundary (Fig. 5 L and M).

The shortened range of extracellular Wg distribution had corresponding effects on the expression of Wg target genes. In wing discs of act>swim RNAi third instar larvae, expression of Sns, a short-range Wg target gene (31), was unaffected (Fig. 5 N and O). However, the range and intensity of Dll, a long-range Wg target gene, was decreased (Fig. 5 P–R and Fig. S10) in wing discs that were age-matched by Sns expression (Fig. S11). Based on the diminished range of extracellular Wg diffusion and shortened long-range signaling activity, we propose that Swim promotes long-range Wg signaling by maintaining Wg solubility in vivo.

Reduction of Swim Leads to a Decrease in the Size of the Wing Disc Pouch. We noticed that swim RNAi larvae reared at higher temperatures had smaller wing disc pouches (Fig. S12A). When we calculated the area of wing disc pouches of age-matched third instar larvae, we found the area of act>swim RNAi wing pouches to be significantly smaller than the area of WT wing pouches from larvae reared at 29 °C (Fig. S12B). This phenotype may be attributed to a defect in Wg distribution, because Wg has mitogenic properties and controls early growth of the wing disc (32–35).

In addition to Wg, other signals influence the growth of the wing disc. Decapentaplegic (Dpp), a Hedgehog target gene, is a well-characterized mitogenic factor important for wing disc growth (33, 36, 37). To assess if the observed decrease in wing pouch size might be because of a putative interaction between Swim and Hedgehog or Dpp pathway components, we assessed phosphorylation of Mothers Against Dpp (MAD) in swim RNAi wing discs. MAD is a cytoplasmic protein that is phosphorylated in response to Dpp signaling (38). Levels of phosphorylated MAD were similar in WT and swim RNAi wing discs from larvae reared at different temperatures (Fig. S12C), indicating that decreasing swim does not impact Dpp activity. However, it remains possible that the small wing pouch phenotype may be caused by an uncharacterized interaction or activity of Swim on pathways unrelated to Wg signaling.

Overexpression of Swim Interferes with Wg Signaling. Wing discs from larvae overexpressing swim displayed an extracellular Wg distribution indistinguishable from the distribution of Wg in WT wing discs (Fig. 6 A–C and Fig. S13); however, there was a measurable difference in the Wg signaling activity between these two genotypes. In WT wing discs, the expression pattern of Dll is graded in direct response to the Wg gradient (Fig. 6D and Fig. S14A). When swim was overexpressed, Dll had a similar range of expression; however, its expression was lower at the DV boundary, and its intensity gradient was more shallow (Fig. 6 E and F and Fig. S14B). This finding suggests that, although Wg diffusion is unaffected in the presence of excess Swim, its signaling activity is impaired, most prominently at or near its site of production (Fig. 6F). In agreement with this observation, Sens expression was also disrupted (Fig. 6 G and H and Fig. S15), and adult wings suffered loss of margin tissue (Fig. 6f). These results complement our in vitro biochemical analyses (Fig. 4) and indicate that excess concentrations of Swim reduce Wg signaling activity, likely by interfering with the ability of Wg to bind its receptors. Altogether, these in vivo experiments show that Swim is necessary for the proper formation of the Wg morphogen gradient.

Discussion

This study shows that Swim, a putative member of the Lipocalin family of extracellular transport proteins, is both necessary and sufficient to potentiate Wg activity in vitro and promote long-range Wg signaling in vivo. We provide evidence that the high-affinity binding interaction between Swim and Wg is palmitate-dependent and that Swim acts, at least in part, to maintain Wg solubility, presumably by concealing the palmitate side chain from the aqueous extracellular environment.

Swim, the Drosophila ortholog of Lcn7, was initially identified as a trace contaminant of our purified Wg preparation. This finding piqued our interest not only because Lipocalins comprise a family of extracellular transport proteins for hydrophobic molecules but also because of the subsequent identification of Lcn7 as a coactivating agent in both mammalian Wnt5a- and Wnt5a-purified protein preparations.

Although it is clear that lipid modification has profound implications on the secretion and activity of Wnt proteins, reports on the role of the palmitate modification using mutant Wnt proteins lacking the necessary acylation sites have been partially contradictory. Our laboratory previously showed that mouse Wnt5aC77A was able to signal in an autocrine manner (14), an observation that was later confirmed by others (13). In contrast, two other groups described mouse Wnt5aΔC107A (39) and Drosophila WgC93A (28) as devoid of any signaling ability. In agreement with reports on Wnt5aC77A, Wnt5aΔC107A, and WgC93A (14, 28, 39), we confirmed that WgC93A is secreted at levels comparable with the levels of WT Wg in vitro. However, although secreted in an active form, WgC93A aggregates in the extracellular space and loses its signaling capabilities. We suspect that this aggregation happens quickly, because activity could be detected in
Addition of a lipid moiety should increase hydrophobicity; therefore, it seems counterintuitive that elimination of Cys-93 acylation promotes Wg protein aggregation. Loss of the palmitate therefore, it seems counterintuitive that elimination of Cys-93 acylation promotes Wg protein aggregation. Loss of the palmitate-dependent manner to maintain Wg solubility and activity is consistent with this model. We propose that loss of the palmitate side chain leads to decreased Wg signaling activity because it abolishes the binding site for this necessary cofactor.

We did not study the biochemistry of the nonpalmitoleoylated form of Wg because loss of the palmitoleate modification inhibits Wg secretion (9, 15), thereby preventing a detailed analysis of the potential role of the palmitoleate moiety in the extracellular Swim–Wg interaction. A competition experiment using free lipid molecules showed that high concentrations of palmitic acid, but not palmitoleic acid, interfere with Swim-Wg binding, suggesting that the palmitoleate moiety may not participate in this interaction. This finding is consistent with the previous findings that the palmitoleate modification is important for Wg activity but not secretion (13, 14, 39). In further agreement with these models, we found that Swim does not affect Wg secretion. Our data suggests that the function of Swim, as it relates to Wg, is exclusively to facilitate extracellular transport after secretion.

Using in vivo RNAi, we showed that reduction of Swim leads to a shortened distribution of extracellular Wg and a consequent...
decrease in the expression of the long-range target gene \textit{dll} but not the short-range target gene \textit{sens}. No phenotype was observed in the embryo in accordance with previous evidence that embryonic development relies solely on short-range Wg signaling (12). However, it is notable that a membrane-tethered form of Wg is sufficient to rescue Wg-null embryos (12). In the absence of Swim, Wg likely remains tethered to the extracellular surface of Wg-secreting cells. Thus, although our data indicate that Swim is not required for short-range Wg signaling, it does not exclude the possibility that Swim may normally mediate this activity.

Overexpressing Swim had no detectable effect on the distribution of extracellular Wg; however, increasing the concentration of Swim impaired Sens expression and affected the gradient of Dll expression. These observations are consistent with our in vitro analysis that show increased concentrations of Swim can outcompete the Wg receptors for access to the ligand. The impairment of the Dll expression gradient and disruption of Sens expression indicate that the consequences of Swim overexpression were most dramatic at the DV boundary. The expression of Sens at the DV boundary requires a high concentration of Wg (31), and we found that a local decrease in the concentration of available Wg interfered with its expression. The expression of Dll requires a relatively low concentration of Wg (11, 12, 40), and competition from an elevated concentration of Swim had a more dramatic effect at the DV boundary where Dll is more highly expressed in response to the high local concentration of Wg. At the periphery of the pouch, where Dll is usually expressed at low levels, there was no measurable effect on Dll expression. We provide evidence that high concentrations of Swim favor the formation of the Wg-Swim complex. However, complex formation occurs in a state of flux, and because a relatively low concentration of Wg is required for the expression of Dll, which is then maintained by additional means (40), a lower rate of Wg-receptor complex formation at the wing disc periphery is sufficient for Dll expression. Taken together, the findings that increasing or decreasing the concentration of Swim in vivo impairs Wg signaling indicate that Swim is important for the proper formation of the Wg morphogen gradient.

There are a number of other factors known to have important roles in the transport of Wg from secreting to responding cells. Two membrane-tethered Heparan Sulfate Proteoglycan molecules, Daily (division abnormally delayed) and Daily-like protein, are essential in shaping the Wg gradient (41–44). In the wing disc the primary role of Daily and Daily-like protein is not to transduce the Wg signal but to stabilize the protein in the extracellular space (42, 45). In the larval neuromuscular junction, there is evidence that Wg travels between cells on exosome-like vesicles containing the Wnt-binding transmembrane protein Evenness Interrupted/ Wntless/Sprinter (46). Lipoprotein particles composed of Lipo-phinor also play a role in the transport of two lipid-linked morphogens, Wg and Hh (18). Depletion of Lipophorin from \textit{Drosophila} imaginal discs leads to a reduction of transcriptional activity of long-range but not short-range Wg target genes (18), suggesting that Lipoprotein particles are important for long-range Wg signaling activity. It is possible that Lipoprotein particles and Swim have similar but parallel roles in facilitating the extracellular transport of Wg, or they could exist in a complex together. Presently, activity assays exhibiting a direct effect of Lipoprotein particles on Wg as an active ligand are lacking, and therefore, the mechanism of how they mediate Wg activity is still unclear.

In summary, Swim is necessary and sufficient to maintain the signaling activity and solubility of Wg in vitro and promote long-range Wg signaling in vivo. The binding of Swim to Wg is mediated by the palmitate side chain, and we propose a mechanism of action that is similar to the mechanism of guanosine nucleotide dissociation inhibitors (GDI) proteins, which bind to prenylated Rab proteins to maintain their solubility during intracellular transit between membranes (47). Although several other mechanisms have been proposed to explain long-range Wg signaling, this mechanism is the first to include a factor that has a measurable effect on Wg as an active ligand. We propose that Swim plays a critical role in the extracellular transport of Wg to maintain formation of a proper signaling gradient.

Materials and Methods

Reagents (Antibodies and Plasmids). All antibodies and plasmids used in this study are described in \textit{SI Materials and Methods}.

Luciferase Reporter Assays. In luciferase reporter assays, 2 x 10^5 reporter cells/well were incubated for 16 h with CM and/or purified protein before luciferase measurement. Swim/Wg reporter assays were performed in serum-free medium.

Protein Purification. Wg was purified as previously described (14). Swim was purified from SwimCM produced by pIB-Swim S2 cells using a three-column FPLC-based purification (Blue Sepharose affinity, Nickel affinity, and Gel Filtration).

Solubility Assay. Purified Wg (0.5 nM) was incubated at room temperature in serum-free media with purified Swim, BSA, l-lactoglobulin, or DFZ2CRD and then centrifuged at 20,000 x g for 45 min at 4°C. Sample buffer was added to the pellet. Blue Sepharose beads were used to pull down the soluble Wg, which was eluted using sample buffer.

Cell Surface Binding Assays. S2 cells transiently transfected with receptor plasmids were incubated with either WgCM or purified Wg before fixing. Immunostaining was performed using standard techniques in detergent-free conditions.
PB5, and cells were imaged on a Zeiss Axioplan2 fluorescence microscope equipped with an Axioimager M2m camera.

**Modified ELISA Protein-Protein Interaction and Competition Assays.** Purified Swim or DFz2CRD was bound to a Nunc Maxisorp plate and then exposed to purified Wg. A saturating amount of Wg antibody (1:50) and 2° antibody (1:2,000, goat anti-Mouse IgG specific alkaline phosphatase, Catalog #A2429; Sigma) were used. AP activity was measured with a FluoroStar Optima fluorimeter. For competition experiments, Wg was preincubated with competitor molecules. Binding affinity and I_{50} values were determined using Prism GraphPad nonlinear regression analysis software.

**Swim in Vitro RNAi.** An established method (48) was used for 52 cell RNAi. A 23-nt region of swim (5′-AAGAGCCATGCCTCATTAGTGCG-3′) was PCR-amplified and cloned into the DMU6-2 pSK vector in a two-step cloning method that created a 5′-3′-3′-3′-23-nt short hairpin.

**Genetics.** Drosophila lines used are as follows: yellow-white WT, actinGAL4/TM6b, nanosGal4/nanosGal4, enGal4/enGal4, UAS-swim, and Trip-cg3074. (77)′ that created a 5′-swim Swim in Vitro RNAi. Terminated using Prism GraphPad nonlinear regression analysis software.

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**Genetics.** Drosophila lines used are as follows: yellow-white WT, actinGAL4/TM6b, nanosGal4/nanosGal4, enGal4/enGal4, UAS-swim, and Trip-cg3074. UAS-swimRNA1-1 and UAS-swimRNA1-2 were generated using a previously described method (29). act-swimRNA1-1 was used to generate all data images. All flies were reared at 23 °C unless otherwise noted.

**Immunohistochemistry.** Imaginal wing discs of third instar larvae reared at 23 °C (unless otherwise noted) were stained for Dll and Sens using standard techniques. The extracellular Wg staining method was previously described (77); Wg antibody was used at a dilution of 1:2.

**Image Analysis.** ImageJ (http://rsb.info.nih.gov/ij/) was used to measure the pixel intensity of Dll or Wg along 11 discrete anteroposterior slices centered around and with relative distance to the DV boundary, covering the area of each wing disc pouch (n = 5 for each experiment). Plots were generated using Prism GraphPad. All images within the same dataset were imaged under identical conditions (extracellular Wg imaged for 900 ms; Dll imaged for 1.3 s).

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SI Materials and Methods

**Plasmids.** For pTub-FLAG-tagged expression construct of WT Wingless (Wg), Wg was amplified using the primers 5′-GCAAGCTTGGGCAAGTCCAGCCAAAGTCC-3′ (forward) and 5′-GCACTTGAATTACAGAGTCGAAGTC-3′ (reverse) and inserted into pFLAG-CMV-1 (Sigma-Aldrich) using HindIII and XbaI. Then, preprotrypsin leader Flag-Wg was amplified by PCR using forward primer 5′-ATTCTAGACCTGTGCTACTTGTTTC-3′ and the aforementioned reverse primer and inserted into pTub using XbaI. The C93A mutation (TGC to GCC) was introduced by overlap extension PCR to create pTub-FWgC93A.

Secreted Wg-interacting molecule (Swim) was PCR-amplified from a cDNA library generated from mRNA from yellow, white flies using the primers 5′-CAGTGGGCCGCCTCGAGGAGTCT-3′ (forward) and 5′-GGTCCGTACACCCGTAGCTAGAGG-3′ (reverse) and then inserted into the pIB-V5/His vector (Invitrogen) using NotI and KpnI sites.

**Reporter Assays.** For transfection experiments, 2 × 10^5 cells/well were transfected with 0.125 μg pSuperTOPflash and 0.0625 μg pIB/LacZ (for normalization), and 0.25 μg each plasmid were done using FuGENE HD and analyzed after 72–96 h. For Fig. 3A, 10^4 Wg reporter cells and Schneider2 (S2) Wg or S2-FWg cells with S2 cells (total of 10^5 cells) were cocultured and analyzed after 24 h. For Fig. 3E, an 8:1:1 mixture of signaling by Wg is impaired by stable shRNA interference (Swissi) of DFz2CRD; 10^4 S2 cells (in 10% serum) were transfected with 0.125 μg pSuperTOPflash and 0.0625 μg pIB/LacZ (for normalization), and 0.25 μg each plasmid were done using FuGENE HD and analyzed after 72–96 h. For Fig. 3E, 5 × 10^4 cells comprised of 50% Wg reporter cells, 25% ligand-producing cells (FWg or FWgC93A), and 25% of either S2 or Swim-overexpressing cells were cocultured in 1% serum and analyzed after 24 h. For Swim/Wg reporter assays, serum-free media, purified Swim [or Swim conditioned media (SwimCM)], and Wg (0.5 μM) were mixed and then added to Wg reporter cells. Luciferase activity was measured by the dual light combination reporter assay system (Applied Biosystems) and a Centro LB960 luminometer (Berthold). β-Gal activity was used to normalize the values. Fold activation was determined by comparing similar samples to those samples that received no Wg. Values are represented with SD of triplicate samples.

**Cell Surface Binding Assays.** S2 cells transiently transfected with pMK-(receptor) were induced by overlap extension PCR to create pTub-FWgC93A. Secreted Wg-interacting molecule (Swim) was PCR-amplified from a cDNA library generated from mRNA from yellow, white flies using the primers 5′-CAGTGGGCCGCCTCGAGGAGTCT-3′ (forward) and 5′-GGTCCGTACACCCGTAGCTAGAGG-3′ (reverse) and then inserted into the pIB-V5/His vector (Invitrogen) using NotI and KpnI sites.

**Protein Purification.** Wg was purified from 6 L WgCM produced by pTub-Wg S2 cells (in 10% serum) as described in the work by Willert et al. (1) in the presence of high salt and high detergent. Swim was purified using a three-column FPLC-based purification: 6 L SwimCM produced by Swim-overexpressing S2 cells were purified over a Blue Sepharose column (bed volume = 143 mL; binding buffer = 150 mM KCl, 20 mM Tris, 1% CHAPS, pH 7.3; elution buffer = 1.5 M KCl, 20 mM Tris, 1% CHAPS, pH 7.3), over a 1-mL Ni^2+^ affinity column (binding buffer = 20 mM phosphate, 0.5 M NaCl, 10 mM Imidazole; elution buffer = 20 mM phosphate, 0.5 M NaCl, 50 mM Imidazole), and then over a Gel Filtration size exclusion column (bed volume = 320 mL; buffer = 1XPBS, 1% CHAPS, 0.5 M NaCl, pH7.3). Resultant fractions were buffer-exchanged into PBS.

**Modified ELISA Protein-Protein Interaction and Competition Assays.** Purified Swim or Drosophila Frizzled2 Cysteine-Rich Domain (DFz2CRD; 1× 10^{-4} Moles) was bound to a Nunc Maxisorp plate O/N at 4 °C. Wells were washed three times with PBS0.1% TX100, blocked with media + 10% FBS/0.1% TX100, and then washed three times with 200 μL PBS/0.1% TX100. Purified Wg was incubated for 1 h at room temperature and then washed four times followed by incubation with Wg monoclonal antibody (1:50, saturating) in block for 1 h at room temperature; then, it was washed four times. The 2 °C antibody (goat anti-mouse IgG specific alkaline phosphatase, Catalog #A2429; Sigma) was added (1:2,000, saturating) in block and incubated for 1 h at room temperature; then, it was washed four times. Colorimetric assay using 50 μL anteroposterior substrate (alkaline phosphatase yellow liquid substrate system for ELISA, Catalog #P7998; Sigma) and a Fluorimeter (FluoStar Optima) was used to assess anteroposterior activity. BSA- or IgG-bound wells were used to assess background for subtraction. Analysis of binding affinity was performed using Prism GraphPad nonlinear regression analysis software.

**In Vitro Secretion Assay.** One microgram pTub-Wg was transfected into 4 × 10^6 cells of each cell type in 2 mL media + 10% serum; 4 dpt, 8 μL CM was loaded onto a polyacrylamide gel for immunoblot analysis.

**Immunohistochemistry.** Imaginal wing discs of third instar larvae reared at 23 °C (unless otherwise noted) were stained for Distalless (Dll) and Senseless (Sens) using standard techniques. For extracellular Wg staining [adapted from the work by Strigini and Cohen (2)], discs were first incubated at 4 °C for 30 min with Wg monoclonal antibody (1:2 in cold media) and then fixed with cold 4% PFA (10 min on ice and 20 min at room temperature). No detergent was used until after fixation.

**Calculation of Wing Disc Pouch Areas.** Wing discs were age-matched at 23 °C (unless otherwise noted) stained for Distalless (Dll) and Senseless (Sens) using standard techniques. For extracellular Wg staining [adapted from the work by Strigini and Cohen (2)], discs were first incubated at 4 °C for 30 min with Wg monoclonal antibody (1:2 in cold media) and then fixed with cold 4% PFA (10 min on ice and 20 min at room temperature). No detergent was used until after fixation.

**Concentrations of Antibodies.** α-Wg monoclonal (4D4) was 1:200 for immunoblots, 1:50 for ELISA, and 1:2 for extracellular Wg staining. Guinea pig α-Sens antibody was 1:1,000 (3). Mouse α-V5 antibody was 1:5,000. Mouse α-DII was 1:400. NFz antibody was 1:2,000. Cy3- and Alexa Fluor 488-conjugated antibodies were 1:400.


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**Fig. S1.** Sequence alignment of Swim orthologs. Amino acid sequence alignment of the predicted Swim orthologs, *Caenorhabditis elegans* (Z81070), *Mus musculus* (Lcn7), *Homo sapiens* (Lcn7), and *D. melanogaster* (Swim) were aligned using the Clustal W program. Identical residues are shaded in black. The somatomedin B domain is represented by a yellow bar above the sequence, and the Lipocalin Signature Motif is represented by a blue bar above the sequence.
Fig. S2. Swim is not autocatalytic. Purified full-length Swim (SwimFL; 49 kDa) was incubated for 24 h in cleavage buffer and analyzed at various time points for the presence of the C-terminal fragment (37 kDa).

Fig. S3. A chimeric Fz::Swim receptor can transduce the Wg signal. (A) A chimeric Fz::Swim receptor was generated by replacing the CRD (the Wg binding domain) of Fz with full-length swim cDNA. fzΔCRD was generated by deleting the CRD. (B) S2 cells were transfected with either WT fz, fz::swim, or fzΔCRD along with the SuperTOPflash reporter, LacZ (for normalization), and either empty pTub (-Wg) or pTub-wg (+Wg). (C) S2 cells transfected with either WT fz, fz::swim, or fzΔCRD (left, red) were treated with WgCM and immunostained for Wg (right, green) cell surface binding.

Fig. S4. FWg and FWgC93A are produced at similar levels. Comparable amounts of secreted proteins are produced by FWg or FWgC93A cells are shown by immunoblotting of whole-cell lysates with anti-Wg.

Fig. S5. BSA cannot interfere with Fz-Wg binding. A constant amount of purified DFz2CRD was immobilized and then exposed to a constant amount of purified Wg. Wg binding was assessed in the presence of increasing concentrations of BSA.
**Fig. S6.** swimRNAi constructs. Schematic of the regions of swim targeted by the RNAi constructs swimRNAi-1 and -2, represented here as lines beneath the corresponding region of swim within its genomic region of chromosome 2R. Gray boxes represent the transcribed regions.

**Fig. S7.** swimRNAi effectively reduces swim transcript levels in larvae. Quantitative RT-PCR analysis of swim transcript levels in WT act>swimRNAi third instar larvae (n = 8).

**Fig. S8.** In vivo reduction of swim leads to a decrease in the extracellular Wg distribution. (A) Quantification of extracellular Wg in WT wing discs and (B) act>swimRNAi wing discs (n = 5). ImageJ was used to measure the pixel intensity of Wg expression of individual slices at defined positions along the anteroposterior axis relative to the dorsoventral boundary, covering the area of the wing pouch (described diagrammatically in Fig. 5H).

**Fig. S9.** Swim does not influence Wg secretion. An ectopic source of wg (pTub-wg) was transfected into swim-overexpressing cells, WT S2 cells, and swim-hairpin cells. CM was collected 96 h posttransfection and visualized with anti-Wg on an immunoblot. The level of Wg secreted into the media was not affected by increasing or decreasing swim expression.
**Fig. S10.** In vivo reduction of *swim* leads to a decrease in the expression of Dll. (A) Quantification of Dll in WT and (B) act>swimRNAi wing discs (*n* = 5). ImageJ was used for quantification as described in Fig. S8.

**Fig. S11.** Age-matching of late third instar larval discs. Dll expression (green) from late third instar larval discs of (A) WT and (B) act>swimRNAi larvae that were age-matched by assessing Sens expression (red; *A’* and *B’*, respectively). Sens expression is only complete at late third instar stages. Here, the act>swimRNAi disc is later third instar relative to the WT disc because the Sens-expressing cells at the dorsoventral boundary have begun to protrude in the z axis.

**Fig. S12.** *swim* RNAi leads to a decrease in wing disc size. (A) Increasing expression of swimRNAi led to a decrease in size of late third instar imaginal wing disc pouches (age-matched by comparing Sens expression). (Scale bars: 100 μm.) (B) Average wing pouch area of WT and act>swimRNAi larvae (*n* = 8 discs; *P* value < 0.01). (C) Phosphorylated-Mad staining of WT and act>swimRNAi larval wing discs.
**Fig. S13.** In vivo overexpression of *swim* has no effect on extracellular Wg distribution. (A) Quantification of extracellular Wg in WT and (B) act>swim wing discs (*n* = 5). ImageJ was used for quantification as described in Fig. S8.

**Fig. S14.** In vivo overexpression of *swim* disrupts the expression gradient of Dll. (A) Quantification of Dll in WT and (B) act>swim wing discs (*n* = 5). ImageJ was used for quantification as described in Fig. S8.
Table S1. Percent lethality in F1 progeny

<table>
<thead>
<tr>
<th>P1 cross</th>
<th>Temperature reared (°C)</th>
<th>18</th>
<th>23</th>
<th>29</th>
</tr>
</thead>
<tbody>
<tr>
<td>act&gt;swim–RNAi-1</td>
<td>0 (n = 29)</td>
<td>58.4 (n = 119)</td>
<td>100 (n = 17)</td>
<td></td>
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<tr>
<td>act&gt;swim–RNAi-2</td>
<td>0 (n = 42)</td>
<td>94.8 (n = 61)</td>
<td>100 (n = 31)</td>
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</table>

Percent lethality resulting from increasing expression of swimRNAi. Expression of swimRNAi was increased by increasing the rearing temperature of developing Drosophila, because the UAS-Gal4 system is temperature-sensitive. Lethality was measured by comparing the number of F1 progeny with or without the TM6B balancer chromosome.

Table S2. Hatching percentage of swimRNAi larvae

<table>
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<th>P1 cross</th>
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<th>29</th>
<th>23</th>
<th>29</th>
</tr>
</thead>
<tbody>
<tr>
<td>act&gt;swim–RNAi1</td>
<td>152</td>
<td>205</td>
<td>181</td>
<td>169</td>
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</tr>
<tr>
<td>Hatched embryos</td>
<td>103</td>
<td>161</td>
<td>122</td>
<td>120</td>
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<tr>
<td>Percent hatched</td>
<td>67.8</td>
<td>78.5</td>
<td>67.4</td>
<td>71.0</td>
<td></td>
</tr>
</tbody>
</table>

To determine the stage at which lethality occurs in swimRNAi larvae reared at 23 °C and 29 °C, embryos were counted before and after hatching into larvae and compared with WT hatching rates.