View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by Elsevier - Publisher Connector

Cell

Wntless, a Conserved Membrane Protein Dedicated to the Secretion of Wnt Proteins from Signaling Cells

Carla Bänziger,[1,2,4] Davide Soldini,[1,4] Corina Schütt,[1,3] Peder Zipperlen,[1] George Hausmann,[1] and Konrad Basler[1,*]
[1] Institut für Molekularbiologie, Universität Zürich, Winterthurerstrasse 190, 8057 Zürich, Switzerland
[2] Ph.D. Program in Molecular Life Sciences, Zürich, Switzerland
[3] The Genetics Company, Inc., Wagistrasse 27, 8952 Schlieren, Switzerland
[4] These authors contributed equally to this work.
*Contact: basler@molbio.unizh.ch
DOI 10.1016/j.cell.2006.02.049

SUMMARY

Cell-cell communication via Wnt signals represents a fundamental means by which animal development and homeostasis are controlled. The identification of components of the Wnt pathway is reaching saturation for the transduction process in receiving cells but is incomplete concerning the events occurring in Wnt-secreting cells. Here, we describe the discovery of a novel Wnt pathway component, Wntless (Wls/Evi), and show that it is required for Wingless-dependent patterning processes in *Drosophila*, for MOM-2-governed polarization of blastomeres in *C. elegans*, and for Wnt3a-mediated communication between cultured human cells. In each of these cases, Wls is acting in the Wnt-sending cells to promote the secretion of Wnt proteins. Since loss of Wls function has no effect on other signaling pathways yet appears to impede all the Wnt signals we analyzed, we propose that Wls represents an ancient partner for Wnts dedicated to promoting their secretion into the extracellular milieu.

INTRODUCTION

The development of complex multicellular organisms relies to large extent on the ability of cells to communicate via extracellular signals. One family of such signaling molecules comprises the Wnt proteins. They direct growth and cell fates in processes as diverse as embryonic segmentation, limb development, and CNS patterning (reviewed by Wodarz and Nusse [1998]). Recent evidence also implicates Wnt signaling in the postembryonic regulation of stem cell number and stem cell differentiation in mammalian skin and colon epithelia as well as in the hematopoietic system (reviewed by Reya and Clevers [2005]). Perturbations in Wnt signaling promote both human degenerative diseases and cancer (reviewed by Logan and Nusse [2004]).

Due to the immense importance of this class of signaling proteins, there is a profound interest to understand how Wnt proteins are synthesized, transported, received, and their signal transduced. A powerful way to reach this goal and identify the components involved is to study Wnt signaling in genetic model systems, such as *Drosophila melanogaster* or *Caenorhabditis elegans*.

A defining member of the Wnt family is the product of the *Drosophila* gene *wingless* (*wg*) (reviewed by Nusse and Varmus [1992]). In the embryo, Wg acts as a short-range inducer to pattern the epidermis: it is secreted by subpopulations of cells within each segment and received by their neighbors, which respond by expressing particular target genes (DiNardo et al., 1988). Loss of Wg function causes a *segment-polarity phenotype*, in which the larval epidermis forms a lawn of ventral denticles and lacks naked cuticle between the segmental denticle belts (Nüsslein-Volhard and Wieschaus, 1980). At later stages, during larval development, Wg also acts at longer distances and in a concentration-dependent manner. In the wing imaginal disc, for example, Wg is produced by cells of the prospective wing margin and spreads symmetrically along the dorsoventral axis to define nested patterns of target-gene expression (Neumann and Cohen, 1997; Strigini and Cohen, 2000; Zecca et al., 1996).

Many of the components of the cellular machinery responsible for transducing Wnt signals from the cell surface to the nucleus have been identified: receptors of the Frizzled and LRP families, the cytosolic proteins Dishevelled, Glycogen Synthase Kinase 3, Axin, APC, and β-catenin, the transcription factors TCF/Lef as well as many of their nuclear cofactors (reviewed by Logan and Nusse [2004]). Less is known about the processes controlling the secretion of Wnt and its movement through the territory between source and target cells. Wnts are secreted as glycoproteins and contain as a hallmark 22 cysteine

residues at conserved positions in their amino acid sequence (Miller, 2002). Some of these cysteine residues might be engaged in the folding of Wnt proteins by disulfide bond formation (Tanaka et al., 2002). Purification and precise biochemical characterization of a Wnt protein succeeded only recently and led to the discovery that the first conserved cysteine residue (C77 in Wnt3a) is palmitoylated (Willert et al., 2003). A candidate acyltransferase to mediate this lipid addition is encoded by the *Drosophila porcupine* (*porc*) and *C. elegans mom-1* genes (Kadowaki et al., 1996; Rocheleau et al., 1997; Hofmann, 2000). This acyltransferase is the only molecularly known pathway component that acts in Wnt-sending cells only. Glypican members of heparan sulfate proteoglycans (Dally and Dally-like in *Drosophila*) also act in Wnt-sending cells, but they mainly function in transporting Wnt proteins as well as other signaling molecules toward target cells (reviewed by Blair [2005]).

To complete the identification of components involved in Wg signaling, we established an in vivo screening system that permits the isolation of Wg pathway genes that have so far resisted isolation by genetic means. Here, we describe the discovery of *wntless* (*wls*), a novel segment-polarity gene in *Drosophila*, and show that it is required for Wg signaling throughout development. In an accompanying report, Bartscherer et al. (2006) refer to this gene as *evenness interrupted* (*evi*). Our characterization of *wls/evi* revealed that it encodes a novel, yet evolutionarily and functionally conserved, transmembrane protein that, intriguingly, is only required in Wg-sending cells. Analogous to Dispatched (Disp), a protein functioning in the release of Hedgehog (Hh) proteins (Burke et al., 1999), Wls is necessary for the secretion of Wnt proteins. However, unlike Disp, we find that this function of Wls is not limited to the lipid-modified form of its target signal. Wls represents only the second component dedicated to the Wnt pathway that acts exclusively in signal-sending cells. Its discovery adds an unanticipated level of complexity that must be factored into our concepts for Wnt-mediated cell-cell communication.

RESULTS

Identification of the *wls* Gene in a Screen for Non-Rate-Limiting Components of the Wg Pathway

As in previous attempts to identify new Wg signaling components, we screened for suppressors of a gain-of-Wg-signaling phenotype in the *Drosophila* eye (*sev-wg*; Brunner et al., 1997). These previous screens were limited to finding dosage-sensitive loci by isolating *dominant* suppressors (Brunner et al., 1997; Kramps et al., 2002). In deliberate contrast to these past efforts, we used here a genetic mosaic set up that also allowed the identification of *recessive* mutations, which suppress the *sev-wg* phenotype only in a homozygous condition (see Experimental Procedures). Two lethal mutations, which fail to complement each other, fulfilled this requirement and are referred to as wls^1 and wls^2. Mosaic animals that in most eye cells lack both wild-type alleles of *wls* show a significant reversion of the rough eye phenotype caused by the *sev-wg* transgene (Figures 1A–1C).

The *wls* Gene Is Required for Wg-Dependent Patterning Processes

To address whether *wls* function is only required for the manifestation of the *wg* overexpression phenotype in the eye or also for patterning processes during normal development, we examined the *wls* mutants in more detail. Animals transheterozygous for wls^1 and wls^2 die as pharate adults with appendage malformations, such as lack of arista and antennal segments, rudimentary legs, and wing-to-notum transformations (Figures 1D, 1E, and see Figure S1 in the Supplemental Data available with this article online). Embryos developing in the absence of both maternal and zygotic *wls* gene function display a segment-polarity phenotype in which the larval epidermis forms supernumerary ventral denticles at the expense of naked cuticle (Figures 1F and 1G). Finally, we analyzed marked *wls* mutant clones in the wing and found these clones to be associated with nicks at the margin of the wing blade (Figures 1H–1J). While the segment-polarity phenotype could be caused by a reduction of either Hh or Wg pathway activity (Nüsslein-Volhard and Wieschaus, 1980), the wing-to-notum transformation and loss-of-wing-margin phenotypes are indicative of reduced Wg activity (Couso et al., 1994; Peifer et al., 1991). Taken together with the suppression of ectopic Wg signaling activity in the eye, described above, our results indicate that *wls* function is required for proper Wg pathway activity at multiple stages of development.

wls Encodes a Novel Seven-Pass Membrane Protein that Is Conserved in Metazoan Genomes

Classical mapping based on meiotic recombination and deletions indicated that the *wls* gene is located within a 240 kb interval that encompasses approximately 50 genes at position 68A/B on the third chromosome. Single-nucleotide polymorphisms were determined in this region by denaturing HPLC and used to fine map the *wls* gene to a critical region of only seven loci. All of these loci were sequenced on DNA prepared from the two mutant *wls* alleles. Only in one gene, *CG6210*, did we detect lesions for both alleles: a 47 bp deletion for wls^1 (causing a frameshift at codon 29) and a C to T transition for wls^2 (changing codon 250 from Pro to Ser). *CG6210* encodes a presumptive 562 residue protein with an N-terminal signal sequence and seven membrane-spanning domains (Figure 1K). A single ortholog of this gene is present in each of the metazoan genomes analyzed, including that of humans (Figure 1K). Apart from the sequence similarities among these orthologous gene products and the hydrophobic stretches presumed to span the lipid bilayer, no sequence motifs could be recognized in the CG6210 protein. Definitive confirmation that *CG6210*

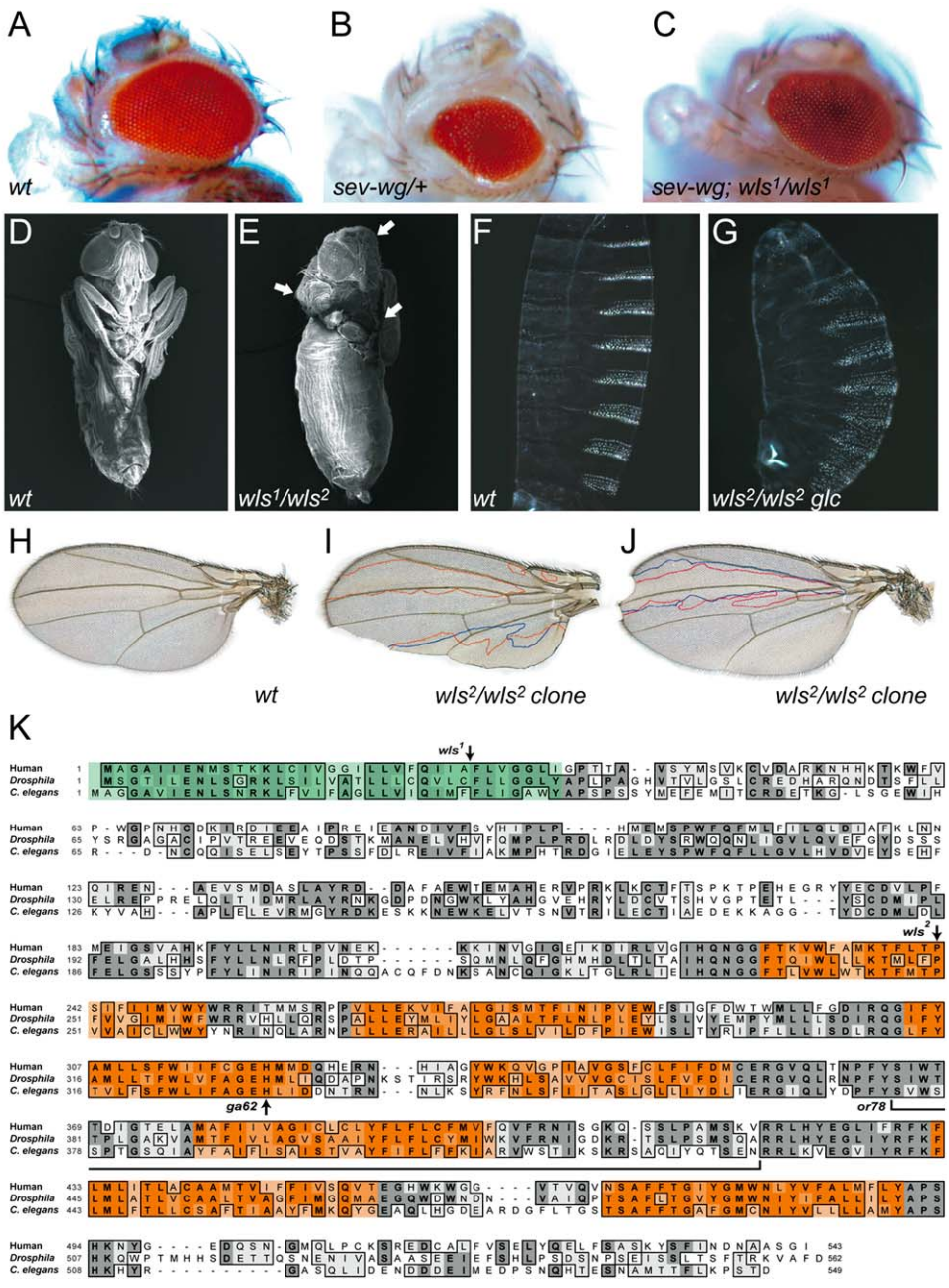

Figure 1. *wls* Is Required for Wg-Dependent Patterning Processes

(A–C) Phenotypes of wild-type (A), *sev-wg*/+ (B), and *sev-wg wls*^{1}/*wls*^{1} (C) eyes.

(D and E) Scanning electron micrographs of wild-type (D) and *wls*^{1}/*wls*^{2} pharate adults (E) removed from the pupal case. Malformations of antennae (top arrow) and legs (right arrow) are shown, as well as a wing-to-notum transformation (left arrow).

(F and G) Cuticle preparations of larvae derived from wild-type parents (F) and from a homozygous *wls*^{2} mutant germline clone (glc) fertilized with *wls*^{2} mutant sperm (G).

(H–J) Adult wings with genetically marked mutant clones. Wings are oriented proximal to the right, anterior up. Clones were marked with *multiple wing hair* (*mwh*). Mutant clones are outlined: red denotes dorsal wing surface; blue denotes ventral surface. Adult wings with *wls* mutant clones show nicks at the margin of the wing blade (if the clone straddles the margin, [I], [J]).

(K) CustalW alignment of Wls protein sequences. Signal sequence, green; transmembrane (TM) domains, orange. The lesions in the *Drosophila* alleles *wls*^{1} and *wls*^{2} are indicated by arrows. *wls*^{1} contains a 47 bp deletion that leads to a frameshift close to the end of the signal sequence (aa 29). *wls*^{2} has an amino acid change from P250 to S within the first TM domain. The *mom-3*/*mig-14* alleles of *C. elegans* exhibit an amino acid exchange from H329 to Y (*ga62*) and a 165 bp deletion which leads to an in-frame loss of amino acids 374 to 428 (*or78*).

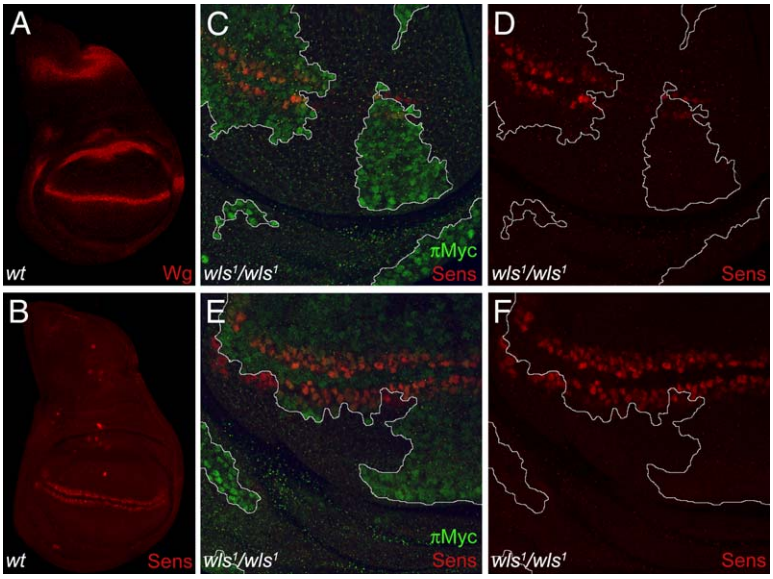

Figure 2. Mosaic Analysis of *wls*

Confocal images of third-instar wing imaginal discs stained with antibodies against the proteins indicated on the lower right of each panel. Genotypes are indicated on the lower left of each panel. Anterior is to the left, dorsal up, and the outlines of mutant clones are traced with a white line.

(A and B) Expression of Wingless (Wg) and Wg target gene Senseless (Sens) at low magnification.

(C–F) High magnifications of large clones of *wls* mutant cells that are marked by the absence of πMyc staining in green (C and E). Sens expression is shown in red.

(C and D) *wls* mutant clones cause loss of Sens expression when they straddle the Wg expression domain; (E and F) Wg target gene expression is unchanged in *wls* mutant clones that do not include the Wg-sending cells.

corresponds to the *wls* gene was obtained with a *tubulinα1* promoter-driven transgene, which rescued viability and all mutant phenotypes caused by *wls* mutations.

wls Is Required in Wg-Sending Rather Than Wg-Receiving Cells

Wls, like the Wg receptor proteins Frizzled and Frizzled-2 (Bhanot et al., 1996), contains seven membrane-spanning regions, raising the possibility that Wls might be involved in receiving and transducing the Wg signal at the cell surface. We sought to address this issue by analyzing the requirement for Wls at high resolution in genetic mosaics. Clones of *wls* mutant cells were generated in the wing primordium and analyzed with respect to their position and expression of the Wg target gene *senseless* (*sens*). A narrow stripe of cells forming the presumptive wing margin secretes Wg and induces on either side the expression of Sens (Figures 2A and 2B), which in turn interacts with proneural genes to define sensory-organ precursor cells (Nolo et al., 2000). We found that *wls* mutant cells fail to express Sens when being part of a clone that straddles the Wg expression domain (Figures 2C and 2D). Unexpectedly, however, we found that cells mutant for *wls* can still express Sens. In all such cases, however, the *wls* mutant cells are close to Wg-secreting cells that are wild-type for *wls* (Figures 2E and 2F). We interpret these findings as genetic evidence that *wls* function is required in Wg-sending rather than Wg-receiving cells.

Retention of Wg in *wls* Mutant Cells

Closer analysis of *wls* clones revealed that Wg-producing cells mutant for *wls* exhibit higher levels of Wg antigen than do wild-type cells (Figures 3A and 3B). The extra Wg protein in mutant cells could either be due to an increased transcription of the *wg* gene or due to a failure to properly secrete and release Wg protein. To discriminate between these two possibilities, we analyzed the activity of the *wg* gene within and the distribution of Wg antigen outside, Wg-producing cells mutant for *wls*. No difference was seen between mutant and wild-type cells when monitoring the activity of a *wg-lacZ* reporter gene inserted into the *wg* locus, suggesting the effect was not on transcription of the *wg* gene (Figures 3C and 3D). However, a clear difference was observed when we examined the distribution of Wg antigen. Normally, Wg is found in punctate structures that decrease in number with increasing distance from the Wg source (Strigini and Cohen, 2000). Strikingly, these punctae are nearly absent in imaginal discs of *wls* mutant animals (Figures 3E–3H). We interpret this shift in the steady-state distribution of Wg antigen, from the outside to the inside of Wg-producing cells, as an indication that *wls* mutant cells retain Wg. In support of this conclusion, we found that ectopic Wg expression, controlled by the heterologous *hsp70* and *sevenless* regulatory elements, is also subject to retention in the absence of *wls* function (Figures 3I–3L).

C. elegans wls Is Required for MOM-2/Wnt-Dependent Polarization of Blastomeres and Corresponds to the Elusive *mom-3/mig-14* Gene

Our conclusion that Wg is retained in *wls* mutant cells can be turned around to argue that *Drosophila* Wls is required for the release of the Wg signal. To address whether this function of Wls is evolutionarily conserved and thus reflects a fundamental relationship between Wls and Wnt signaling, we sought to analyze the roles of the human (see below) and *C. elegans* orthologs. The first Wnt-dependent process in nematode development occurs at the four and eight cell stages, when the Wnt signal MOM-2 orients the mitotic spindles and division planes of blastomere cells (Thorpe et al., 2000). To test if the *C. elegans* Wls protein (encoded by ORF *R06B9.6*) is

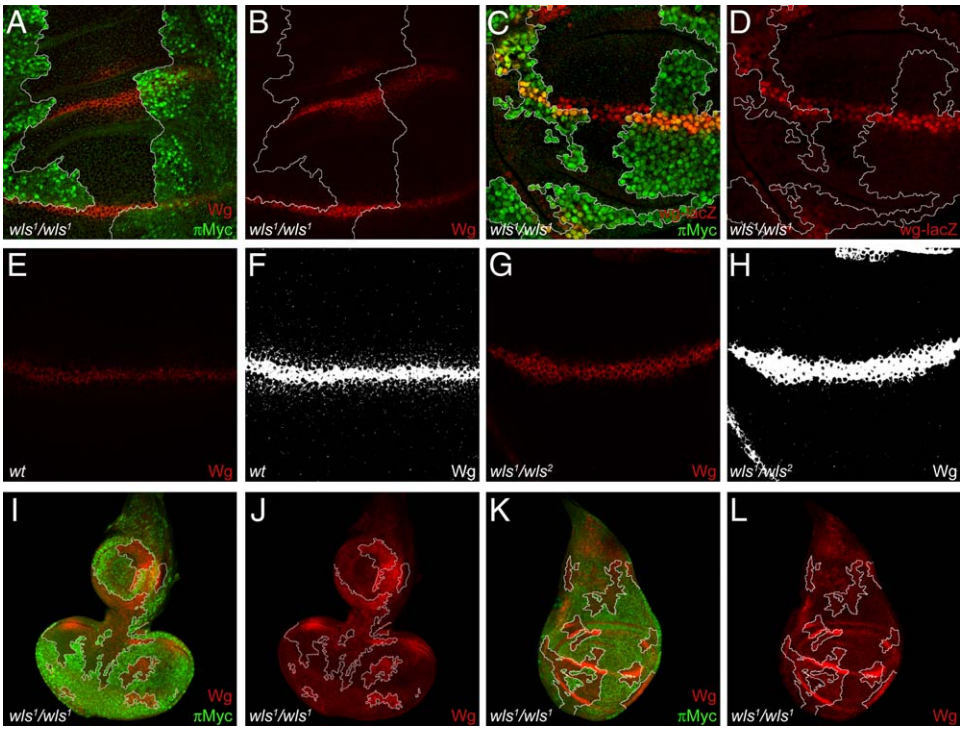

Figure 3. Retention of Wg in *wls* Mutant Cells

(A and B) Wg antigen distribution (red) is upregulated in a cell-autonomous manner in *wls* mutant cells expressing *wg*. Mutant tissue is marked by the absence of πMyc (green).

(C and D) *wg-lacZ* expression (in red, $wg^{lacZro216}$) is unchanged in *wls* mutant cells, suggesting that the higher levels of Wg antigen observed in (A) and (B) are not due to increased transcription of *wg*. Mutant tissue is marked by the absence of πMyc (green [C]).

(E–H) Wg gradient in wild-type (E and F) and *wls* mutant (G and H) wing discs. The wild-type (*wt*) and mutant (wls^1/wls^2) wing discs are shown at identical, nonsaturating recording conditions to the left in red (E and G). To better visualize Wg antigen found in punctate structures, contrast-enhanced images are shown to the right in white (F and H). Note that at the site of Wg production, less Wg antigen is detected in wild-type (E) compared to mutant (G) discs. Instead, wild-type discs show a substantial amount of Wg antigen in punctate structures (that decrease in number with increasing distance from the source [H]); this gradient is almost undetectable in wing discs mutant for *wls* (G). The stripe of Wg-sending cells appears more narrow in wild-type discs because wild-type *wg*-expressing cells at the edge of the stripe show only very low levels of intracellular Wg antigen (as verified by double staining for *wg-lacZ* and Wg antigen).

(I–L) Ectopically expressed Wg (red) accumulates in *wls* mutant tissue (marked by the absence of the green πMyc in eye-antennal discs [I and J] and wing discs [K and L]). Wg was expressed in a subset of eye disc cells by means of the *sevenless* enhancer of the *sev-wg* transgene and in addition in all cells by means of the *hsp70* promoter present in this transgene (see Experimental Procedures).

involved in this process, *R06B9.6* dsRNA was injected into the gonads of wild-type adult hermaphrodites (see Experimental Procedures). Embryos derived from such animals invariably showed a spindle-orientation defect in the ABar cell (Figure 4D; 10 out of 10 recorded embryos). *RNAi (mom-2)* embryos (8 out of 8) were analyzed in parallel and showed the same ABar spindle misorientation phenotypes (Figure 4B). Thus, the *C. elegans wls* gene, like its *Drosophila* ortholog, is required for developmental events that are controlled by a Wnt ligand.

In addition to *mom-2/wnt*, three other genes had previously been identified to play a role in orienting the spindle and cell-division plane of the ABar blastomere, i.e., *mom-1*, *mom-3/mig-14*, and *mom-5* (Thorpe et al., 1997). It was postulated that together the products of these genes form a Wnt signaling cassette for determining cytoskeletal polarity in certain early blastomeres (Rocheleau et al., 1997). This notion was borne out by the demonstration that *mom-1* encodes the *C. elegans* homolog of Porcupine, and *mom-5* a Frizzled-related protein (Rocheleau et al., 1997). *mom-3* was mapped to a narrow region of chromosome 2 (20.41 ± 0.914 cM), but has so far withstood molecular identification. Intriguingly, ORF *R06B9.6* is located in the same region of chromosome 2, raising the possibility that *R06B9.6* might be *mom-3*. We sequenced the *R06B9.6* coding region from three different *mom-3* alleles and found that the *mom-3(or78)* allele (Thorpe et al., 1997) has a 165 bp deletion removing the fifth transmembrane domain of the presumptive R06B9.6 protein, and the *mom-3/mig-14(ga62)* allele (Eisenmann and Kim, 2000) has a C-to-T transition causing an amino acid exchange at position 329 (His to Tyr, see Figure 1K). We failed to detect an alteration of the *R06B9.6* coding region in the *mom-3(mu71)* allele

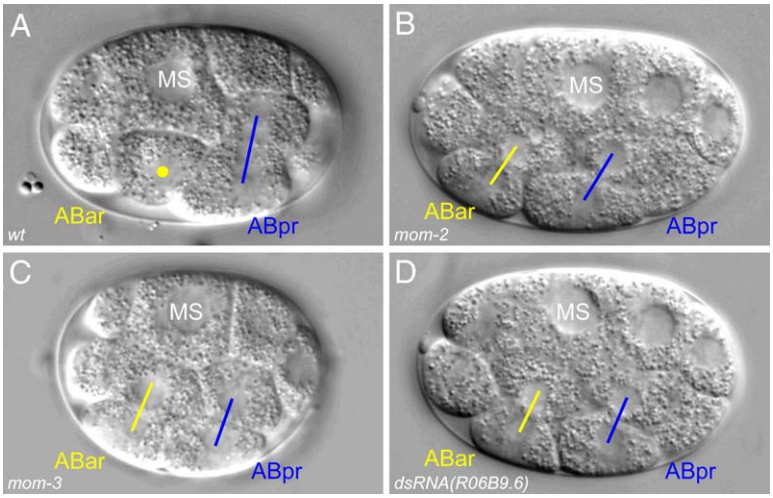

Figure 4. RNAi against the *C. elegans wls* Ortholog Phenocopies the ABar Spindle Orientation Defect of *mom-2* and *mom-3* Mutant Embryos

Embryos are shown anterior to the left and ventral up.

(A) Wild-type embryo, the ABar spindle (yellow) is oriented perpendicular to the spindle of ABpr (blue), i.e., perpendicular to the optical section shown (indicated by the yellow dot).

(B) In *RNAi(mom-2)*-injected animals, the two spindles are in parallel orientation.

(C and D) In *mom-3(or78)* mutant embryos (C) as well as in *RNAi(R06B9.6)* embryos (D), the ABar and ABpr spindles are also in parallel orientation.

(Harris et al., 1996); however, the mutation may lie in a region important for controlling the transcription of the gene. When we compared *RNAi(R06B9.6)* embryos (10 out of 10, see above) and *mom-3(or78)* embryos (9 out of 9), we observed equivalent ABar spindle misorientation phenotypes in both classes (Figures 4C and 4D). We thus conclude that *mom-3* encodes the *C. elegans* ortholog of Wls. The site of action of *mom-3*, like that of *mom-2*, had previously been mapped by mosaic analysis to the P2 blastomere (Thorpe et al., 1997), indicating that in nematode embryos, as in *Drosophila*, Wls function is required in the Wnt-sending cell.

Human Wls Is Required for Intercellular Signaling by Wnt3a

For studying the release of and response to human Wnt proteins, we used embryonic kidney cells (HEK-293T) transfected with a Wnt3a expression vector and cocultured these producer cells with responder cells transfected with the transcriptional TCF/Lef reporter plasmid *superTOPFLASH* (Veeman et al., 2003; Figure 5A). Wnt3a from producer cells caused an approximately 15-fold induction of Luciferase levels in the responder population (Figure 5B). Two different siRNAs ($siRNA^{hWLS\text{-}A}$, $siRNA^{hWLS\text{-}B}$) were validated by RT-PCR for their effectiveness to knock down the expression of the endogenous *hWLS* gene in HEK-293T cells. Independent transfection of both resulted in an 85% decrease of *hWLS* transcripts (not shown; $siRNA^{hWLS\text{-}B}$ was mostly used and is henceforth referred to as *sihWLS*, but $siRNA^{hWLS\text{-}A}$ showed the same effects). Treatment of responder cells with *sihWLS* or treatment of producer cells with siRNA against *GFP* (*siGFP*) did not appreciably affect the outcome of the Wnt signaling assay. In contrast, when *sihWLS* was cotransfected into Wnt3a producer cells, there was no induction, and Luciferase levels were comparable to control

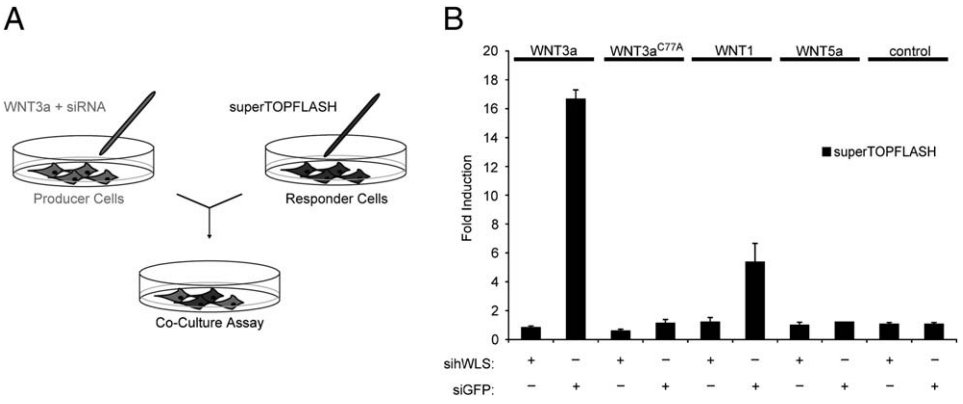

Figure 5. Wls Functions in Wnt Signaling between Human HEK-293T Cells

(A) To generate signal-producing cells, one population of cells was transfected with Wnt3a (and siRNA against *hWLS* and *GFP*, respectively). Responder2 cells were prepared by transfection with the reporter plasmid *superTOPFLASH*.

(B) *superTOPFLASH* assay in HEK-293T responder cells. Relative Luciferase levels (ratio between firefly luciferase activity from *superTOPFLASH* and *Renilla* luciferase activity from *CMV* promoter) were normalized to the negative control (*pcDNA3* alone) and the resulting "fold induction" is shown. The error bars shown represent standard deviations.

experiments in which the producer cells were transfected only with empty vector (Figure 5B). These results demonstrate that human Wls plays an essential role in Wnt-mediated cell-to-cell communication in vitro; furthermore, they specify the Wnt-sending cells as the site of hWls action. This is in agreement with the results obtained independently in *Drosophila* and *C. elegans*, indicating that the function of Wls orthologs in Wnt signaling is evolutionarily conserved.

Wnt3a Reaches Neither the Culture Medium nor the Surface of Wls-Depleted Cells

The failure of *sihWLS*-treated producer cells to activate the Wnt pathway in responder cells could be due to a failure to secrete Wnt3a or the secretion of an inactive Wnt3a. To clarify this, we assayed whether the levels of Wnt3a protein released by producer cells depend on Wls function. The levels of Wnt3a (C-terminally tagged with a V5 epitope) were reduced at least 20-fold in the supernatant of *sihWLS*-treated cells when compared with the control (Figure 6A). Thus, although we cannot exclude an additional effect for Wnt3a *activity*, this result indicates that knockdown of *hWLS* drastically abrogates the secretion of Wnt3a.

Because the *wls* gene appears to function in mammalian and *Drosophila* systems for Wnt secretion and mutant cells fail to deliver Wnt ligand to surrounding cells, we refer to this gene as *wntless*, as a description of the mutant situation.

To test whether Wnt3a protein levels are reduced because Wnt3a does not reach the cell surface or because it reaches the surface but fails to be released into the medium, we assayed the surface levels of Wnt3a-V5 by immunocytochemistry. As a control for this procedure, we used cells expressing N- or C-terminally tagged CD2 proteins, of which we detected only the N-terminally tagged form under nonpermeabilizing conditions (Figures 6B–6I). Wnt3a-V5 protein was present on the surface of control-treated producers (Figure 6C) but undetectable on the surface of *sihWLS*-treated cells (Figure 6B). There was no difference in the distribution of N-terminally tagged CD2 protein (Figures 6F and 6G). A potential caveat in this experiment could stem from the vast difference in Wnt3a concentration in the culture medium; individual cultured cells may exhibit detectable Wnt3a ligand on their surface when it is highly abundant in the supernatant, irrespective of whether they actually produced it or not. However, independent marking of transfected cells indicated that only those cells that express Wnt3a show surface staining (data not shown). From these results, we can conclude that Wnt3a requires Wls for the passage to the surface. Our results do not exclude that Wls has a second role in the release of Wnts from the surface membrane.

Specificity of Wls Action: Dedication to Wnt Signals

While the above-described experiments provide solid grounds for a scenario in which Wls is required for the secretion of Wg and Wnt3a, we have not yet addressed the degree of specificity of Wls activity on protein secretion. First, we will consider the extent to which Wls is required for the secretion of other, non-Wnt proteins, and then we will explore the question of selectivity within the Wnt family.

A plausible explanation for the effects of RNAi-mediated knockdown of *hWLS* in HEK-293T cells would be a global role for Wls in vesicular trafficking and protein secretion. To address this possibility, we monitored the secretion of a biologically inert control protein, SS^{HA}-$dTNF^{\Delta N}$ (a truncated from of *Drosophila* TNF with a hemagglutinin signal sequence, see Experimental Procedures), in the presence and absence of Wls. As shown in Figure 6J, SS^{HA}-$dTNF^{\Delta N}$ levels were not reduced in response to *sihWLS* treatment of HEK-293T cells, arguing against a general requirement of Wls in protein secretion. Support for this conclusion can also be derived from the surface levels of HA-CD2 protein (see above, Figures 6F and 6G), which did not differ in the presence or absence of Wls. A further telling control was carried out with Sonic hedgehog (Shh), a protein that, like Wnt3a, is lipid modified and used in intercellular communication. Shh protein levels in the supernatant of HEK-293T cells were unaffected by *WLS* knockdown (Figure 6K). A related experiment was performed in imaginal tissues of *Drosophila*, where we monitored Hh protein levels in mutant cell clones. While cells mutant for *disp* showed a clear accumulation of Hh ligand, no difference in Hh levels was detectable in cells mutant or wild-type for *wls* (data not shown).

The most informative experiment relating to the specificity issue was the rescue of *wls* mutant animals by a transgene combination in which *wls* expression is restricted to Wg-sending cells. A *UAS-wls* transgene alone failed to ameliorate the *wls* mutant situation. However, *UAS-wls* driven by a *wg-Gal4* driver (expression is restricted to cells that normally produce Wg; Figure S2) resulted in a high-fraction of phenotypically normal, viable adult animals (> 80%). These animals developed correctly patterned appendages, despite the fact that numerous signaling pathways, such as those governed for example by Dpp, EGF, Notch, and Insulin, had to operate essentially in the "absence" of *wls* gene function (see also Figure S3). Moreover, these animals effectively demonstrate that Wls is only required in Wg-*sending* cells, strengthening our hypothesis based on genetic mosaics that Wls is dispensable in Wg-*receiving* cells.

To begin to address to what extent other Wnt family members require Wls function for secretion, we analyzed Wnt1, another Wnt member that activates the canonical β-catenin signaling pathway, and Wnt5a, a representative Wnt operating in a "noncanonical" manner (Torres et al., 1996). For Wnt1, we observed a 5-fold pathway induction in the coculture assay, which was abolished by treatment of producer cells with *sihWLS* (Figure 5B). The protein levels of Wnt1-HA secreted into the medium of cultured cells were measurably reduced when Wls was knocked down (Figure 6E). Wnt5a protein was secreted in low amounts into the supernatant of HEK-293T cells but not following treatment with *sihWLS* (Figure 6M). These

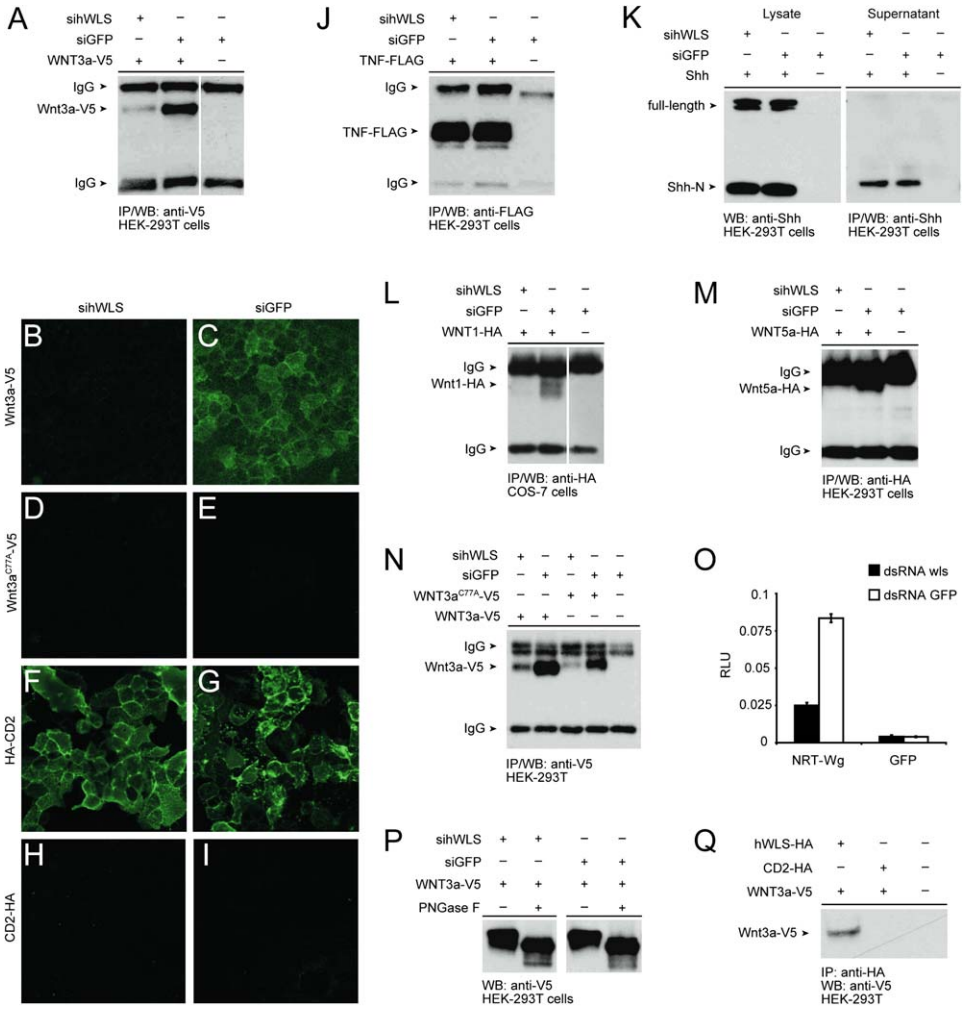

Figure 6. Secretion of Wnt3a Is Impaired upon Knockdown of *hWLS*

(A) The level of Wnt3a-V5 in the supernatant of HEK-293T cells is strongly decreased by depletion of hWls.

(B–I) Cell surface stainings of HEK-293T cells. Wnt3a-V5 cannot reach the cell surface of cells cotransfected with *sihWLS* (B), whereas cotransfection of *siGFP* leads to detectable levels of Wnt3a-V5 at the cell surface (C). Wnt3a^{C77A}-V5 is neither detectable at the surface of cells treated with *sihWLS* (D) nor *siGFP* (E).

(F–I) HA-CD2 is detected on the cell surface (F and G) while CD2-HA is not (H and I); CD2-HA can be readily detected by conventional staining (data not shown). Surface levels of HA-CD2 are not affected by *sihWLS* (F).

(J) Secretion of SS^{HA}-dTNF$^{\Delta N}$ (TNF-FLAG) is not affected by depletion of Wls.

(K) Lysate and culture medium of HEK-293T cells cotransfected with Shh and *sihWLS* show no alteration in production and secretion of Shh compared to controls (*siGFP*).

(L) *sihWLS* abolishes secretion of Wnt1-HA into the medium of COS-7 cells.

(M) Application of *sihWLS* strongly decreases secretion of Wnt5a-HA compared to *siGFP*.

(N) Although secretion of Wnt3a^{C77A}-V5 is not as efficient as wild-type Wnt3a-V5, it is also affected by depletion of Wls.

(O) Signaling activity of NRT-Wg is reduced in Kc167 cells treated with dsRNA against Wls (signaling levels were quantified by coculture with S2 cells stably transfected with a Wg-responsive LEF luciferase reporter system).

(P) Neither levels nor glycosylation status of Wnt3a-V5 are affected by *sihWLS*. Shown are HEK-293T cell lysates with or without treatment by N-Glycosidase F.

(Q) Wnt3a molecularly interacts with hWls, but not with an unrelated membrane protein (CD2) in HEK-293T cells. Shown are immunoprecipitations probed with anti-V5 antibody.

results suggest that Wls is required by most, if not all, Wnt family members. Support for this view can be derived from the genetic requirement of the *C. elegans wls* ortholog *mom-3*; not only is it involved in early blastomere polarization, which is governed by the Wnt MOM-2 (see above; Rocheleau et al., 1997; Thorpe et al., 1997), it is also required for the specification of a subset of vulval and male spicule precursor cells (Eisenmann and Kim, 2000;

Herman et al., 1995; Jiang and Sternberg, 1999) and for the migration of Q neuroblasts along the anterior/posterior body axis (Harris et al., 1996), processes that are governed by the Wnt ligands LIN-44 and EGL-20, respectively (reviewed by Korswagen [2002]). In *Drosophila*, none of the seven Wnts, with the exception of Wg, has been found to be essential for viability. However, DWnt2 and DWnt4 are required for cell-fate determination and cell motility in developing testes and ovarioles, respectively (Kozopas et al., 1998; Cohen et al., 2002). Intriguingly, we observed that *wls* mutant animals, rescued by the *wg-Gal4/UAS-wls* transgene combination described above, exhibit testicular and ovarian phenotypes as well as male and female sterility (data not shown), consistent with the view that also in *Drosophila* multiple Wnts depend on Wls for their function.

Wls Is Also Required for the Secretion of a Nonpalmitoylatable Form of Wnt3a

Mature Wnt ligands are lipid-modified through the addition of a palmitate group to the first conserved cysteine residue (C77 in Wnt3a; Willert et al., 2003). We used a nonpalmitoylatable form of Wnt3a (Wnt3a^{C77A}) to explore the relationship between Wls function and palmitoylation of Wnt proteins. Wnt3a^{C77A} is secreted into the supernatant by HEK-293T cells (Figure 6N). However, unlike wild-type Wnt3a, it is not detectable on the surface of producing cells (Figures 6D and 6E), presumably owing to the lack of its membrane tether. Moreover, Wnt3a^{C77A} lacks signaling activity when tested on responder cells (Figure 6N). Interestingly, when *hWLS* was knocked down by RNAi, we found that the levels of Wnt3a^{C77A} protein in the supernatant were reduced (Figure 6B). Hence, secretion of Wnt3a^{C77A} still depends on Wls activity, and it is therefore not the palmitate-modification that necessitates the action of Wls for the secretion of wild-type Wnt3a.

We also addressed whether Wls function can be bypassed by rerouting Wnt proteins along a potentially different secretory pathway. For this we used a form of Wg devoid of its signal sequence but fused instead to the C terminus of the type I membrane protein Neurotactin (Nrt-Wg; Zecca et al., 1996). In a coculture assay similar to the one depicted in Figure 5A, Kc167 cells expressing Nrt-Wg showed a significant decrease in signaling activity upon treatment with dsRNA against *wls* (Figure 6O). Cell-surface staining indicated that this reduction in signaling activity was accompanied, and hence possibly caused by, a diminished cell-surface pool of Nrt-Wg (data not shown). Thus, Wg is not liberated from its Wls dependency by adopting the secretory conventions of a type I transmembrane protein; in contrary, even unrelated proteins such as Neurotactin become dependent on Wls function when fused to Wg.

Localization and Physical Interaction of Wls and Wnt3a in the Secretory Pathway

To address whether Wls acts directly on Wnt proteins to promote their secretion or whether it exerts its effect indirectly, we started to investigate the subcellular localization and physical interaction of Wg/Wnt3a and Wls. For immunocytochemistry, we used *Drosophila* imaginal discs, HEK-293T cells, and Vero cells, another kidney cell line derived from African green monkeys that offers higher spatial resolution. *Drosophila* and human Wls proteins were tagged with an HA tag at their C termini; this modification does not affect Wls activity as a *tubulinα1-wls-HA* transgene fully rescues *wls* mutant animals (data not shown). Human Wls-HA was present at high levels in punctae that correspond to the Golgi apparatus, and at lower levels in a population of vesicles between the Golgi and the cell surface (Figures 7A–7F). Wnt3a-V5 was distributed in a broader pattern, reflecting the entire secretory pathway (Figures 7G–7L). No difference was observed in the distribution of Wnt3a-HA protein upon treatment of such cells with *sihWLS* (Figures 7M–7R; the susceptibility of Vero cells to *sihWLS* was validated by the demonstration that also in Vero cells, the knockdown of Wls expression prevents surface localization of Wnt3a). Consistent with this finding, Wnt3a proteins isolated from lysed sending (Wnt3a-producing) cells exhibited the same glycosylation status, irrespective of whether such cells were treated with siRNA against *hWLS* (Figure 6P).

Subcellular colocalization of Wg and Wls was observed in vesicular structures of *Drosophila* imaginal disc cells (Figure S3), raising the possibility that the two proteins physically interact. To test this assumption, hWls-HA or as a negative control CD2-HA was immunoprecipitated from lysed HEK-293T cells coexpressing Wnt3a-V5. Western blot analysis revealed that Wnt3a-V5 was present in the hWls-HA precipitates but absent from the CD2-HA samples (Figure 6Q). Thus, we conclude that although the knockdown of endogenous Wls does not cause a detectable difference in the intracellular distribution of Wnt3a, the two proteins colocalize and physically interact late in the secretory pathway. Since neither localization nor extracellular levels of Wnt3a were detectably changed upon coexpression with extra hWls (not shown), endogenous hWls levels cannot be rate limiting for Wnt3a secretion, a conclusion expected by the design of the genetic screen that led to the isolation of Wls in the first place.

DISCUSSION

Cell-cell communication by Wnt proteins controls a multitude of fundamental patterning processes during animal development. Numerous and diverse layers of regulatory mechanisms have been built into this signaling system to control strength, range, and precision of Wnt-mediated outputs. Most of the layers known so far cover steps within the signal-receiving cell. Others have been found to affect the movement of Wnt proteins between their source and target cells. Here, we report the discovery of a novel aspect of Wnt signaling in the signal-sending cells. In a genetic screen to isolate novel non-rate-limiting pathway components, we identified Wls, a seven-pass membrane protein that is dedicated to promoting, and possibly

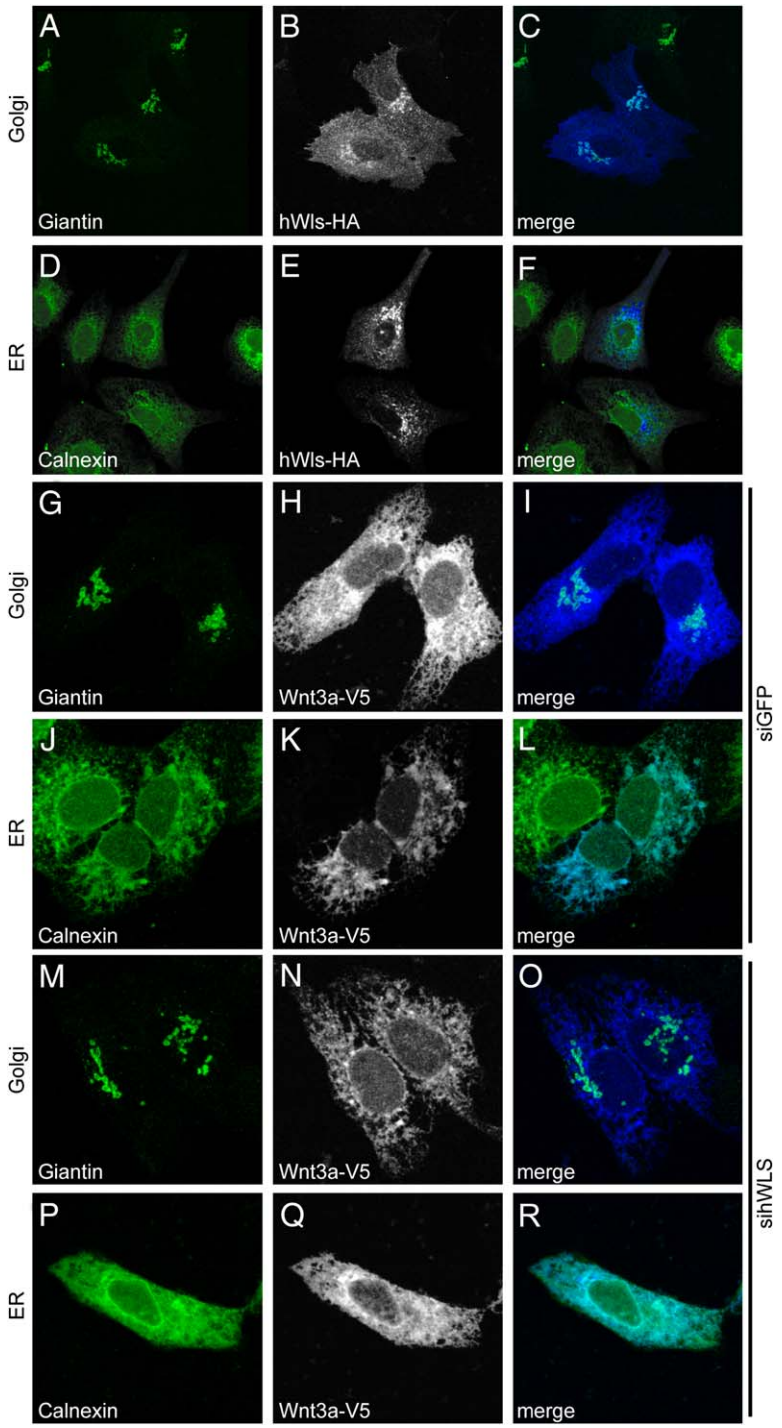

Figure 7. Localization of hWls and Wnt3a in Vero Cells

As a marker for the Golgi apparatus and the ER we used Giantin (in green [A, G, and M]) and Calnexin (in green [D, J, and P]), respectively. The merge of the organelle staining and hWls-HA or Wnt3a-V5 staining is shown in the panels to the right (hWls-HA and Wnt3a-V5 staining in blue).

(A–F) Localization of hWls-HA in untreated Vero cells. hWls-HA is present mainly in the Golgi apparatus.

(G–L) In control cells treated with *siGFP* Wnt3a-V5 is mainly localized to the ER but also present in the Golgi.

(M–R) Knockdown of Wls does not lead to a detectable accumulation of Wnt3a-V5 in the ER or Golgi.

controlling, the secretion of Wnt proteins. One immense but exciting hurdle to the elucidation of the Wls function at a molecular level is that the process of Wnt secretion is essentially a black box. Below, we discuss what our results have revealed about this function and what some of the many unanswered questions are that the discovery of Wls raises.

Activity versus Secretion of Wnt Proteins

Our genetic experiments showing that Wls is only required in Wg-sending cells, together with the finding that Wnt3a activity requires Wls function in cultured producer but not responder cells, placed Wls upstream of the Wnt/Wg receptors LRP/Arrow and Frizzled and the downstream signal-transduction cascade. These results on their own

could not discriminate between the possibilities that in the absence of Wls Wnt ligands are sent out in an *inactive* form or are *not sent out* at all. However, the accumulation of Wg protein in *wls* mutant cells, together with the severe reduction of Wg/Wnt levels in tissue or medium surrounding mutant cells, indicated that the prime cause of the *wls* mutant phenotype must be a shortage of Wg/Wnt ligands in their target field. In the absence of Wls, Wg/Wnt proteins are predominantly retained in the producing cells and are thus unable to move in adequate quantities to nearby cells.

Some residual Wg-dependent patterning still occurs in *wls* mutants, such as reduced expression of the low-threshold Wg target gene *Distal-less* (Figure S4). Hence, a small fraction of Wg protein must be escaping, below the limits of immunohistochemical detection. Consistent with this assumption, we found that *wls* homozygous mutant larvae did not survive a 50% reduction of Wg expression (by removal of one *wg* gene copy). All these observations suggest that the function of Wls is to ensure the efficient secretion of Wnts at physiologically effective levels. It is important to emphasize that these results do not exclude an additional role of Wls in conferring *activity* to Wnt ligands, a role that could be masked by the secretion phenotype.

Transport to the Surface versus Release from the Surface

The identification of a multitransmembrane protein required for the secretion of Wnt signaling proteins is reminiscent of the analogous discovery of Disp (Burke et al., 1999). In the absence of Disp, Hh signals are not secreted from cells and are unable to reach neighboring cells. In both pathways, the ligands are lipid-modified and tightly associated with cell membranes, yet are able to move over considerable distances in vivo (reviewed by Nusse [2003]). The predominant view of how Disp functions is that it releases Hh ligands from their membrane association. In apparent contrast to Disp, Wls seems to function earlier in the pathway, as it is already required for the *transport* of Wnt ligands to the cell surface, not just for their *release* from the membrane.

Other proteins implicated in the release of signaling proteins are the glypicans Dally-like (Dlp) and Dally (reviewed by Blair [2005]), which belong to the family of heparan sulfate proteoglycans (HSPGs). Both Dlp and Dally bind Wg protein (Baeg et al., 2001; Strigini and Cohen, 2000), and if their function is perturbed (by removing Sulfateless, an enzyme required for their synthesis) Wg protein no longer accumulates at the surface of Wg-secreting cells and animals exhibit *wg* loss-of-function phenotypes (Lin and Perrimon, 1999). These phenotypes are superficially similar to those caused by the lack of Wls function, raising the possibility that Wls might function like Dlp and Dally or play an essential role in their biosynthesis. However, in embryos with perturbed glypican function, Wg protein is undetectable at the surface of secreting cells because it is not stabilized there and released too readily (Pfeiffer et al., 2002). By contrast, when Wls function is perturbed, Wg/Wnt protein is poorly released, and its extracellular concentration is diminished rather than increased. Moreover, glypicans play a role in the movement of Wg from cell to cell (Franch-Marro et al., 2005), a role we could exclude for Wls by our analysis of genetic mosaics. Together, our results indicate that Wls is required for proper intracellular transport of Wg/Wnt proteins to the cell surface.

Why Do Wnts Require a Seven-Pass Membrane Protein to Reach the Cell Surface?

If Wnt proteins use the general mode of intracellular conveyance, like other proteins destined for secretion, they may need special assistance to do so. For example, it is possible that Wnt polypeptides require a molecular chaperone function to reach a particular structural state for proceeding in vesicular transport from the ER to the Golgi apparatus. Wls might provide such a chaperone function, and in its absence Wnt ligands might not be admitted to public transportation. An intriguing parallel to such a chaperone function for Wnt signals is the recently identified chaperone for Wnt receptors: Boca and its mammalian homolog Mesd (Culi and Mann, 2003; Hsieh et al., 2003) are specifically required for the folding and stability of a subset of the low-density lipoprotein receptor (LDLR) family, which also comprises the Wg and Wnt receptors Arrow and LRP5/6. A high degree of client specificity is not unprecedented for molecular chaperone systems, but several observations argue against the view that Wls is a Wnt chaperone. First, for most chaperone systems analyzed, unfolded client proteins are targeted for degradation, rather than accumulation, in the absence of chaperone function. Second, the movement of such improperly folded polypeptides is blocked early along the secretory pathway, at the level of the ER, a phenotype we could not observe in cells lacking Wls function. And finally, the predominant location of Wls in the Golgi apparatus does not support a function in the ER for this protein.

Wnt proteins are posttranslationally modified by the addition of a palmitate group at a conserved cysteine residue (Willert et al., 2003). A candidate enzyme for catalyzing this lipid modification is encoded by the *Drosophila porc* gene. It is possible that lipid-modified Wnt proteins require a dedicated mechanism for their secretion and that Wls is part of such a mechanism. In principle, it is also possible that Wls is required together with Porc to palmitoylate Wg protein. However, Wls and Porc localize to different compartments of the secretory pathway, and our findings in mammalian cells indicate that $Wnt3a^{C77A}$, which lacks the palmitoylation site (Willert et al., 2003), is still subject to regulation by Wls. Therefore, although we cannot rule out a functional overlap between Porc and Wls function, we favor the view that Wls is acting *subsequent* to Porc in an aspect of the secretion of Wnt proteins that does not center on their palmitate modification.

Wls as an Intracellular Wnt Router or Rerouter?

Two recent observations that may relate to the mechanism of Wls action are particularly interesting. One concerns the discovery that a significant fraction of Wg protein inside secreting cells of the embryo is present in endocytic vesicles and sent back to the surface of such cells (Pfeiffer et al., 2002). The precise function of this ligand recycling is not yet known, but it may allow Wg protein to gain access to cellular compartments from which standard secretory proteins are excluded. A role that could be envisaged for Wls is to relieve Wg from this "holding pattern" and allow it to ride public transportation out of the cell. Hence, loss of *wls* function would cause an abnormally high amount of Wg to cycle in this holding pattern, accompanied by a corresponding deficit in the extracellular milieu.

The other observation concerns lipophorin particles in *Drosophila* and the notion that such particles may serve as vehicles for the movement of lipid-modified ligands such as Wg or Hh (Panakova et al., 2005). An intriguing possibility would be if Wls is required for loading Wg protein onto such particles in a certain cellular compartment to which lipophorin particles and Wg have common access.

In a variation of these themes, Wnt proteins may have to cycle between different secretory or endocytic compartments before they can be secreted as active proteins. This cycling may endow them with posttranslational modifications or permit their charging onto lipophorin particles. Wls might be required to liberate Wnt proteins from this cycle and permit their subsequent secretion as mature signals.

A Dedicated Partner for all Wnts

Whatever the precise molecular mechanism by which Wls allows Wnt proteins to be secreted, it is remarkable that Wls functions so specifically on Wnt proteins. In a diverse set of experiments in which Wls function was reduced, spatially restricted, or abolished, we failed to detect any effects of Wls on other secreted proteins or cellular processes. Equally astonishing is the observation that Wls is required for all members of the Wnt family we or others have analyzed in this respect. In nematode and vertebrate systems, a subset of Wnt ligands has been found to signal via "noncanonical" routes to control the polarities of individual cells; examples for such ligands are MOM-2 and Wnt5a, respectively. Our findings suggest that both polarity Wnts, as well as Wnt signals activating canonical transduction pathways, depend on Wls function for their respective activities. This apparent "pan-Wnt promiscuity" argues for a fundamental relationship between Wls and structural aspects, as opposed to functional properties, of Wnt proteins. The presence of a single Wls ortholog in all metazoan genomes, and its absence in unicellular phyla and plants, indicates that Wnt signaling always depended on, and hence coevolved with, Wls function.

EXPERIMENTAL PROCEDURES

Genetic Screen and Mapping of *wls* Alleles

Males of the genotype *y w ey-flp* ; *FRT80 sev-wg* / *TM6b[y$^+$]* were mutagenized with EMS and crossed to *y w ey-flp* ; *M(3)i^{55} FRT80* / *TM2[y$^+$]* to generate animals with eyes composed largely of homozygous mutant cells. 38,000 progeny were screened; suppressors were re-screened, balanced, and subjected to complementation analysis. The *3L3$^{su20.53}$* (*wls^1*) and *3L3$^{su20.54}$* (*wls^2*) alleles were mapped by meiotic recombination, complementation assays with deficiencies, and SNP analysis (details available on request). Denaturing high-performance liquid chromatography (DHPLC) was used on a WAVE MD system (from Transgenomics) to discriminate between homo- and heteroduplices. Based on genetic criteria, both *wls* alleles appear to be genetic nulls.

Transgenes and siRNA

GenBank accession numbers for the *Drosophila* and human *wls* genes are *DQ305404* and *DQ323735*, respectively. For all *Drosophila wls* constructs, splice form A was used, based on EST *GH01813. hWLS* was made by a combination of the two incomplete human ESTs *BC007211* and *BQ669223* (Invitrogen) and expressed in *pcDNA3*, tagged with either HA or V5 tag. Wnt3a was also equipped C-terminally with either tag; Wnt1 and Wnt5a carried a C-terminal HA epitope. N- and C-terminally tagged rat CD2 constructs will be described elsewhere. SS^{HA}-dTNF$^{\Delta N}$ consists of a biologically inert form of the *Drosophila* TNF homolog Eiger (amino acids 212 to 415) carrying N-terminally the signal sequence from Influenza Hemagglutinin and a FLAG tag.

Target sequences for the siRNAs were as follows:

hWLS-A, CGG CGT CAC AGT CCA AGT GAA;
hWLS-B, CAC GAA TCC CTT CTA CAG TAT.

dsRNA Injections and Recordings of *C. elegans* Cell Divisions

Generation of dsRNA, injections, and recordings are described by Zipperlen et al. (2001). Primers used for synthesis of *R06B9.6* dsRNA are as follows:

5′-*CGGCGCGCCCCGATTTGCCGGAAGTTTTC*-3′;
5′-*AGGCCGGCCTCCTCAACTCACGAGAAGCT*-3′.

Primers used for synthesis of *mom-2* dsRNA are as follows:

5′-*CGTAATACGACTCACTATAGGGAGATTTGTTTGGGCTGGATGCTC*-3′;
5′-*CGTAATACGACTCACTATAGGGAGAAAGATCCAGCGGATGGAGA*-3′.

Cell Culture and Immunocytochemistry

HEK-293T and Vero cells were grown under standard conditions and transfected with calcium phosphate and Lipofectamine Plus, respectively. Cells were processed for immunocytochemistry 40 hr after transfection. Epitope-tagged proteins were detected with rabbit anti-HA (ICL) and mouse anti-V5 (Invitrogen). Anti-Calnexin was used as a marker for the ER, anti-Giantin as a marker for the Golgi complex. For coculture assays, cells were mixed 24 hr after transfection and incubated for 48 hr before they were lysed and processed for measuring firefly and *Renilla* luciferase expression by the Dual-Luciferase reporter assay (Promega) on a Wallac Victor-2.

Kc167 cells were transfected with constructs encoding Nrt-Wg, HA-Wg, or GFP; 3 hr after transfection, cells were split into wells for mixing with dsRNA against either Wls or GFP. Forty-eight hours after transfection, these cells were mixed with Wg S2 reporter cells and cultured for further 24 hr. Cells were harvested and Luciferase values measured.

Immunoprecipitations and Western Analysis

Two days after transfection, culture medium was incubated overnight at 4°C with Protein G Sepharose beads (Amersham Biosciences) and antibody (1:1000). Cells were lysed in RIPA buffer and Benzonase for 1 hr on ice. Beads were washed four times in TBS prior to elution with NuPAGE LDS sample buffer and NuPAGE sample reducing agent (Invitrogen) for 10 min at 96°C. The following antibodies were used: mouse anti-V5 (Invitrogen), mouse anti-HA.11 (BAbCo), mouse anti-FLAG (Sigma), and mouse anti-Shh-N (5E1). For detection of Shh on blots, the anti-Shh antibody MAB464 (R&D Systems) was used at a dilution of 1:2000. For the IP of Wnt1-HA and Wnt5a-HA, 100 μg/ml Heparin was added to cells before the medium was harvested. PNGase F treatment was performed according to the manufacturer's instructions (New England Biolabs).

Clonal Analysis and Rescue Experiments

Germline clones were obtained from animals of the following genotype:

y w hsp70-flp ; wls^2 FRT80 / FRT80 P[ovoD] x *wls^2 FRT80 / TM6b*.

Wing clones were analyzed in the following animals:

y w *hsp70-flp ; mwh^1 wls^2 FRT80 / P[hsp-πMyc, w^+] $M(3)i^{55}$ FRT80*.

Disc clones were obtained from the following larvae:

y w *hsp70-flp ; wls^1 FRT80 / P[hsp-πMyc, w^+] $M(3)i^{55}$ FRT80*.

Clones were heat-shock-induced at 48 hr after egg laying (1 hr at 37°C). Larvae carrying the *hsp70-πMyc* transgene were heat-shocked a second time (1.5 hr at 37°C) prior to dissection. This second heat shock also induced Wg expression in larvae that additionally carried the *sev-wg* construct (which contains the promoter of the *hsp70* gene between the two *sevenless* enhancers and the *wg* coding region). Third-instar larvae were fixed and stained by standard techniques. Antibodies were mouse monoclonal anti-Myc 9E10, guinea pig anti-Sens (Nolo et al., 2000), mouse monoclonal anti-Dll (gift from I. Duncan), and Alexa 488 and 594 fluorescent secondary antibodies (Molecular Probes).

For the rescue experiments, transheterozygous wls^1/wls^2 animals, which carried either a *tubulinα1-wls* transgene or a combination of a *UAS-wls* and *wg-Gal4 ND382* or *S180* (Gerlitz et al., 2002), were generated. Most *wg-Gal4* insertions are also *wg* loss-of-function alleles and genetically interact with *wls*, causing wing-to-notum phenotypes at high frequency.

Supplemental Data

Supplemental Data include four figures and can be found with this article online at http://www.cell.com/cgi/content/full/125/3/509/DC1/.

ACKNOWLEDGMENTS

We thank E. Arenas, S. Cohen, A. Helenius, J. Kitajewski, R. Moon, and R. Nusse for antibodies or plasmids, A. Hajnal and C. Mosimann for advice and comments on the manuscript, and P. Geuking, M. Hediger, C. Mosimann, and D. Sirena for help with experiments and figures. This project was supported by the Swiss National Science Foundation and the Kanton of Zürich.

Received: December 21, 2005
Revised: February 9, 2006
Accepted: February 27, 2006
Published: May 4, 2006

REFERENCES

Baeg, G.H., Lin, X., Khare, N., Baumgartner, S., and Perrimon, N. (2001). Heparan sulfate proteoglycans are critical for the organization of the extracellular distribution of Wingless. Development *128*, 87–94.

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

Bhanot, P., Brink, M., Samos, C.H., Hsieh, J.C., Wang, Y., Macke, J.P., Andrew, D., Nathans, J., and Nusse, R. (1996). A new member of the frizzled family from Drosophila functions as a Wingless receptor. Nature *382*, 225–230.

Blair, S.S. (2005). Cell signaling: wingless and glypicans together again. Curr. Biol. *15*, R92–R94.

Brunner, E., Peter, O., Schweizer, L., and Basler, K. (1997). pangolin encodes a Lef-1 homologue that acts downstream of Armadillo to transduce the Wingless signal in Drosophila. Nature *385*, 829–833.

Burke, R., Nellen, D., Bellotto, M., Hafen, E., Senti, K.A., Dickson, B.J., and Basler, K. (1999). Dispatched, a novel sterol-sensing domain protein dedicated to the release of cholesterol-modified hedgehog from signaling cells. Cell *99*, 803–815.

Cohen, E.D., Mariol, M.C., Wallace, R.M.H., Weyers, J., Kamberov, Y.G., Pradel, J., and Wilder, E.L. (2002). DWnt4 regulates cell movement and focal adhesion kinase during *Drosophila* ovarian morphogenesis. Dev. Cell *2*, 437–448.

Couso, J.P., Bishop, S.A., and Martinez Arias, A. (1994). The wingless signalling pathway and the patterning of the wing margin in Drosophila. Development *120*, 621–636.

Culi, J., and Mann, R.S. (2003). Boca, an endoplasmic reticulum protein required for wingless signaling and trafficking of LDL receptor family members in *Drosophila*. Cell *112*, 343–354.

DiNardo, S., Sher, E., Heemskerk-Jongens, J., Kassis, J.A., and O'Farrell, P.H. (1988). Two-tiered regulation of spatially patterned engrailed gene expression during Drosophila embryogenesis. Nature *332*, 604–609.

Eisenmann, D.M., and Kim, S.K. (2000). Protruding vulva mutants identify novel loci and Wnt signaling factors that function during Caenorhabditis elegans vulva development. Genetics *156*, 1097–1116.

Franch-Marro, X., Marchand, O., Piddini, E., Ricardo, S., Alexandre, C., and Vincent, J.P. (2005). Glypicans shunt the Wingless signal between local signalling and further transport. Development *132*, 659–666.

Gerlitz, O., Nellen, D., Ottiger, M., and Basler, K. (2002). A screen for genes expressed in Drosophila imaginal discs. Int. J. Dev. Biol. *46*, 173–176.

Harris, J., Honigberg, L., Robinson, N., and Kenyon, C. (1996). Neuronal cell migration in C. elegans: regulation of Hox gene expression and cell position. Development *122*, 3117–3131.

Herman, M.A., Vassilieva, L.L., Horvitz, H.R., Shaw, J.E., and Herman, R.K. (1995). The *C. elegans* gene lin-44, which controls the polarity of certain asymmetric cell divisions, encodes a Wnt protein and acts cell nonautonomously. Cell *83*, 101–110.

Hofmann, K. (2000). A superfamily of membrane-bound O-acyltransferases with implications for wnt signaling. Trends Biochem. Sci. *25*, 111–112.

Hsieh, J.C., Lee, L., Zhang, L., Wefer, S., Brown, K., DeRossi, C., Wines, M.E., Rosenquist, T., and Holdener, B.C. (2003). Mesd encodes an LRP5/6 chaperone essential for specification of mouse embryonic polarity. Cell *112*, 355–367.

Jiang, L.I., and Sternberg, P.W. (1999). Socket cells mediate spicule morphogenesis in Caenorhabditis elegans males. Dev. Biol. *211*, 88–99.

Kadowaki, T., Wilder, E., Klingensmith, J., Zachary, K., and Perrimon, N. (1996). The segment polarity gene porcupine encodes a putative multitransmembrane protein involved in Wingless processing. Genes Dev. *10*, 3116–3128.

Korswagen, H.C. (2002). Canonical and non-canonical Wnt signaling pathways in Caenorhabditis elegans: variations on a common signaling theme. Bioessays *24*, 801–810.

Kozopas, K.M., Samos, C.H., and Nusse, R. (1998). DWnt-2, a Drosophila Wnt gene required for the development of the male reproductive tract, specifies a sexually dimorphic cell fate. Genes Dev. *12*, 1155–1165.

Kramps, T., Peter, O., Brunner, E., Nellen, D., Froesch, B., Chatterjee, S., Murone, M., Zullig, S., and Basler, K. (2002). Wnt/wingless signaling requires BCL9/legless-mediated recruitment of pygopus to the nuclear beta-catenin-TCF complex. Cell *109*, 47–60.

Lin, X., and Perrimon, N. (1999). Dally cooperates with Drosophila Frizzled 2 to transduce Wingless signalling. Nature *400*, 281–284.

Logan, C.Y., and Nusse, R. (2004). The Wnt signaling pathway in development and disease. Annu. Rev. Cell Dev. Biol. *20*, 781–810.

Miller, J.R. (2002). The Wnts. Genome Biol. *3*, REVIEWS3001.

Neumann, C.J., and Cohen, S.M. (1997). Long-range action of Wingless organizes the dorsal-ventral axis of the Drosophila wing. Development *124*, 871–880.

Nolo, R., Abbott, L.A., and Bellen, H.J. (2000). Senseless, a Zn finger transcription factor, is necessary and sufficient for sensory organ development in *Drosophila*. Cell *102*, 349–362.

Nusse, R. (2003). Wnts and Hedgehogs: lipid-modified proteins and similarities in signaling mechanisms at the cell surface. Development *130*, 5297–5305.

Nusse, R., and Varmus, H.E. (1992). Wnt genes. Cell *69*, 1073–1087.

Nüsslein-Volhard, C., and Wieschaus, E. (1980). Mutations affecting segment number and polarity in Drosophila. Nature *287*, 795–801.

Panakova, D., Sprong, H., Marois, E., Thiele, C., and Eaton, S. (2005). Lipoprotein particles are required for Hedgehog and Wingless signalling. Nature *435*, 58–65.

Peifer, M., Rauskolb, C., Williams, M., Riggleman, B., and Wieschaus, E. (1991). The segment polarity gene armadillo interacts with the wingless signaling pathway in both embryonic and adult pattern formation. Development *111*, 1029–1043.

Pfeiffer, S., Ricardo, S., Manneville, J.B., Alexandre, C., and Vincent, J.P. (2002). Producing cells retain and recycle Wingless in *Drosophila* embryos. Curr. Biol. *12*, 957–962.

Reya, T., and Clevers, H. (2005). Wnt signalling in stem cells and cancer. Nature *434*, 843–850.

Rocheleau, C.E., Downs, W.D., Lin, R., Wittmann, C., Bei, Y., Cha, Y.H., Ali, M., Priess, J.R., and Mello, C.C. (1997). Wnt signaling and an APC-related gene specify endoderm in early *C. elegans* embryos. Cell *90*, 707–716.

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

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

Thorpe, C.J., Schlesinger, A., Carter, J.C., and Bowerman, B. (1997). Wnt signaling polarizes an early *C. elegans* blastomere to distinguish endoderm from mesoderm. Cell *90*, 695–705.

Thorpe, C.J., Schlesinger, A., and Bowerman, B. (2000). Wnt signalling in Caenorhabditis elegans: regulating repressors and polarizing the cytoskeleton. Trends Cell Biol. *10*, 10–17.

Torres, M.A., Yang-Snyder, J.A., Purcell, S.M., DeMarais, A.A., McGrew, L.L., and Moon, R.T. (1996). Activities of the Wnt-1 class of secreted signaling factors are antagonized by the Wnt-5A class and by a dominant negative cadherin in early Xenopus development. J. Cell Biol. *133*, 1123–1137.

Veeman, M.T., Slusarski, D.C., Kaykas, A., Louie, S.H., and Moon, R.T. (2003). Zebrafish prickle, a modulator of noncanonical Wnt/Fz signaling, regulates gastrulation movements. Curr. Biol. *13*, 680–685.

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

Wodarz, A., and Nusse, R. (1998). Mechanisms of Wnt signaling in development. Annu. Rev. Cell Dev. Biol. *14*, 59–88.

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

Zipperlen, P., Fraser, A.G., Kamath, R.S., Martinez-Campos, M., and Ahringer, J. (2001). Roles for 147 embryonic lethal genes on C.elegans chromosome I identified by RNA interference and video microscopy. EMBO J. *20*, 3984–3992.