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Secretion of Wnt Ligands Requires Evi, a Conserved Transmembrane Protein

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SUMMARY

Wnt signaling pathways are important for multiple biological processes during development and disease. Wnt proteins are secreted factors that activate target-gene expression in both a short- and long-range manner. Currently, little is known about how Wnts are released from cells and which factors facilitate their secretion. Here, we identify a conserved multipass transmembrane protein, Evenness interrupted (Evi/Wls), through an RNAi survey for transmembrane proteins involved in *Drosophila* Wingless (Wg) signaling. During development, *evi* mutants have patterning defects that phenocopy *wg* loss-of-function alleles and fail to express Wg target genes. *evi*'s function is evolutionarily conserved as depletion of its human homolog disrupts Wnt signaling in human cells. Epistasis experiments and clonal analysis place *evi* in the Wg-producing cell. Our results show that Wg is retained by *evi* mutant cells and suggest that *evi* is the founding member of a gene family specifically required for Wg/Wnt secretion.

INTRODUCTION

Many patterning decisions during development are controlled by morphogens, secreted signaling molecules that inform cells about their relative position within a tissue. It has been proposed that these morphogens form gradients with the highest activity acting on cells immediately adjacent to the source of signal production, leading to differential target-gene expression across the tissue (reviewed in Cadigan, 2002). Signaling molecules of the Wnt, Hedgehog (Hh), and TGF-β families can spread over many cell diameters to activate target-gene expression in cells far away from the source of production (Lawrence and Struhl, 1996; Zecca et al., 1996). Consequently, the localized expression of such a morphogen can function as an organizing center that determines the growth and patterning of the surrounding tissue (Basler and Struhl, 1994; Diaz-Benjumea and Cohen, 1994).

Drosophila Wingless (Wg) is a founding member of the Wnt family and plays multiple roles during embryonic and larval development (reviewed in Seto and Bellen, 2004; Logan and Nusse, 2004). Wg activates a signal-transduction cascade by binding to its receptor complex consisting of members of the Frizzled (Fz) protein family and the coreceptor LRP6/Arrow (Arr) (Bhanot et al., 1996; Wehrli et al., 2000). Upon activation of Dishevelled (Dsh) and recruitment of Axin to the plasma membrane, the Armadillo (Arm; the *Drosophila* homolog of β-catenin) degradation complex is inhibited, and Arm accumulates in the cytoplasm. After translocation to the nucleus, Arm binds Pan/TCF proteins and activates transcription of Wg-responsive target genes (Axelrod et al., 1998; Boutros et al., 1998; Brunner et al., 1997; Riese et al., 1997; van de Wetering et al., 1997; reviewed in Logan and Nusse, 2004).

Wg can activate its target genes in a short- or long-range manner. In wing imaginal discs, Wg is secreted by a narrow stripe of cells at the dorsoventral border and activates the expression of short-range targets, such as *senseless* (*sens*), up to 4 cells away from the source (Nolo et al., 2000). Expression of long-range targets, such as *distal-less* (*dll*) or *vestigial* (*vg*), requires lower levels of Wg and occurs as far as 30 cell diameters away from the signal-producing source (Lawrence et al., 1996; Neumann and Cohen, 1997; Zecca et al., 1996). Cells at the dorsoventral border that receive high levels of Wg give rise to the wing margin. Expression of *sens*, for example, is necessary for the generation of sensory organs such as the wing-margin bristles (Nolo et al., 2000).

How molecules with high membrane affinities such as Hh and Wg can be secreted and function over such long distances remains poorly understood. Similarly to Hh proteins, Wg is attached to membranes presumably via covalent lipid modifications (Willert et al., 2003; reviewed by Mann and Beachy, 2004). Palmitoylation has been proposed to target Wg to secretory vesicles that deliver the ligand to specialized microdomains at the apical plasma membrane where it is secreted (Zhai et al., 2004). In addition to posttranslational modifications, endocytosis and degradation play a role in regulating the extracellular levels of Wg available for signaling (Piddini et al., 2005; Panakova et al., 2005; Marois et al., 2006).

Here, we present *evenness interrupted* (*evi*), a conserved segment polarity gene required for Wg signaling

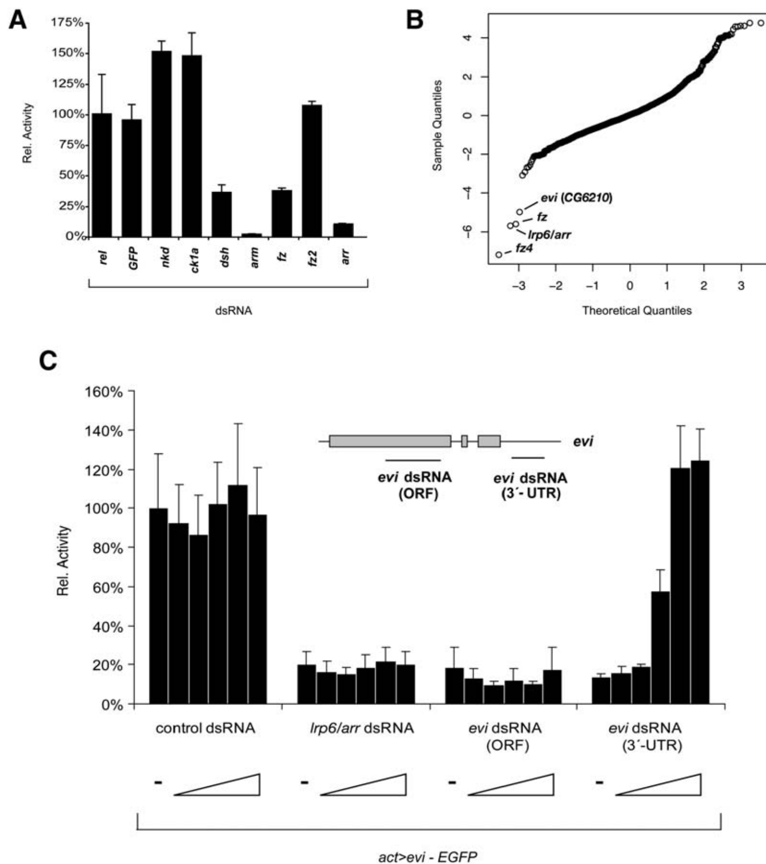

Figure 1. *evi* Is a Novel Regulator of Wg Signaling in *Drosophila* Cells

S2R$^+$ cells (A and C) or Kc_{167} macrophage-like cells (B) were transfected with the Wg-responsive *pLEF$_7$-luc* reporter, *pAc-LEF-HA*, *pAc-wg*, and *pRp128-RL*. Cells were incubated for 5 days with dsRNAs against the indicated target genes prior to analysis of reporter-gene activity.

(A) dsRNA against known positive (*dsh*, *arm*, *fz*, and *lrp6/arr*) and negative regulators (*nkd* and *ck1α*) of Wg signaling changes reporter activity relative to negative control dsRNAs (*rel* and *GFP*). Relative activity is defined by the Wg-mediated induction of firefly luciferase expression normalized against the constitutively expressed *Renilla* luciferase. Data are represented as mean ± SD.

(B) *evi* was found in an RNAi screen for transmembrane proteins involved in Wg signaling. Graphical representation of the screening results, as shown with a plot of normally distributed quantiles against actual pathway screening results. Values represent z scores.

(C) Expression of *evi-EGFP* lacking the endogenous 3′UTR rescues the *evi* RNAi phenotype. RNAi against *lrp6/arr* or *evi* with two independent RNAs reduces reporter activity compared to a *lacZ* control dsRNA. *pAc-evi-EGFP* (act>evi-EGFP) rescues the RNAi phenotype caused by the *evi* 3′UTR dsRNA in a dose-dependent manner. Data are represented as mean ± SD. The target regions of both *evi* dsRNAs are depicted schematically in the inset.

in cultured cells and in vivo. *evi* was found in an RNAi survey for transmembrane proteins involved in Wg signaling. We show that Evi is a member of a previously uncharacterized protein family and that its human homolog is required for Wnt signal transduction in human cells. In *Drosophila*, clonal analysis demonstrates that *evi* is required for Wg but not for Hh target-gene expression. Epistasis studies and clonal analysis place *evi* in the Wg-producing cell. We show that Wg is retained by *evi* mutant cells, suggesting that Evi is a member of a family of transmembrane proteins that specifically control the secretion of Wnt ligands. In a related paper in this issue of *Cell*, Bänziger et al. (2006) refer to this gene as *wntless* (*wls*).

RESULTS

Identification of *evenness interrupted*, a Regulator of Wg Signaling

To identify novel transmembrane proteins required for Wnt/Wg signaling, we performed an RNAi survey by examining the phenotypes of ~2300 putative transmembrane proteins encoded by the *Drosophila* genome in a cell-based assay for Wg signaling activity (see Experimental Procedures). S2R$^+$ and Kc_{167} cells were transfected with the Wnt-responsive LEF_7-luc reporter (Giese et al., 1995) and a Wg expression plasmid (*pAc-wg*), which induces the expression of the reporter gene approximately 40-fold relative to basal activity (data not shown). RNAi against positive regulators of Wg signaling reduces reporter-gene expression, while RNAi targeting negative regulators increases reporter activity (Figure 1A). Using dsRNAs from a genome-wide RNAi library (Boutros et al., 2004; Hild et al., 2003), we assessed Wg signaling phenotypes in Kc_{167} cells after depletion of transcripts by RNAi. While the majority of dsRNAs used in the survey did not decrease reporter activity, four dsRNAs caused a significant reduction of the Wnt reporter signal. Among them, we found members of the family of frizzled receptors, *fz* and *fz4*, and the coreceptor *lrp6/arr*. In addition, we identified *CG6210* (Figure 1B), which we named *evenness interrupted* (*evi*) due to its wing-margin phenotype (see below). The requirement of *evi* for Wg signaling was also confirmed in S2R$^+$ cells (Figure 1C).

To confirm that the RNAi phenotype of *evi* dsRNA was specifically due to *evi* depletion, we designed an independent dsRNA against the *evi* 3′UTR. Both ORF and 3′UTR dsRNAs reduce reporter activity by approximately 80% compared to control dsRNAs. Transfection of an Evi-EGFP expression construct (*pAc-evi-EGFP*) lacking the endogenous *evi* 3′UTR rescues the loss-of-function phenotype caused by the *evi* 3′UTR dsRNA in a dose-dependent manner. Transfection of *pAc-evi-EGFP* did not increase basal reporter activity and was not sufficient to rescue the phenotype of *lrp6/arr* dsRNA or the *evi* ORF

dsRNA (Figure 1C). Together, these experiments show that the *evi* RNAi phenotype is specific and that *evi* is required for Wg signaling in cultured cells.

evi Encodes a Conserved Multipass Transmembrane Protein

The *evi* gene encodes two alternative transcripts for a 562 aa and a 594 aa protein. Database searches revealed that the predicted full-length protein belongs to a family of highly conserved proteins present in the genomes of metazoans from *C. elegans* to man. Interestingly, and in contrast to other factors in the Wnt signaling pathway, both vertebrate and invertebrate genomes appear to encode for only a single gene homologous to *evi* (Figure 2A). The *Drosophila* and human Evi proteins share 43% overall sequence identity. Sequence analysis predicts that *evi* encodes a type II transmembrane protein with eight membrane-spanning regions (Figure 2B).

To examine the subcellular localization of Evi, an *Evi-EGFP* transgene was expressed in wing imaginal-disc cells. As shown in Figure 2C and in Figure S1A in the Supplemental Data available with this article online, Evi-EGFP localizes primarily to the plasma membrane in vivo. Localization of Evi-EGFP at the plasma membrane was also seen in cultured S2R$^+$ cells and human HEK293T cells (data not shown and Figure S1B).

To determine whether the requirement of *evi* for Wnt signal transduction is conserved, we depleted the homolog of *evi* (*hevi*) in human cells. HEK293T cells were transfected with a Wnt reporter as previously described (van de Wetering et al., 1997); expression plasmids for mouse Wnt1, mouse Fz8, or human LRP6; and siRNAs against β-*catenin*, *lrp6*, *hevi*, or *GFP*. As shown in Figure 2D, siRNAs directed against *hevi* reduce reporter activity to levels similar to those observed by siRNAs against β-*catenin* or *lrp6*. These experiments indicate that *hevi* is required for Wnt signaling in humans and that the function of *evi* in Wnt signaling is conserved.

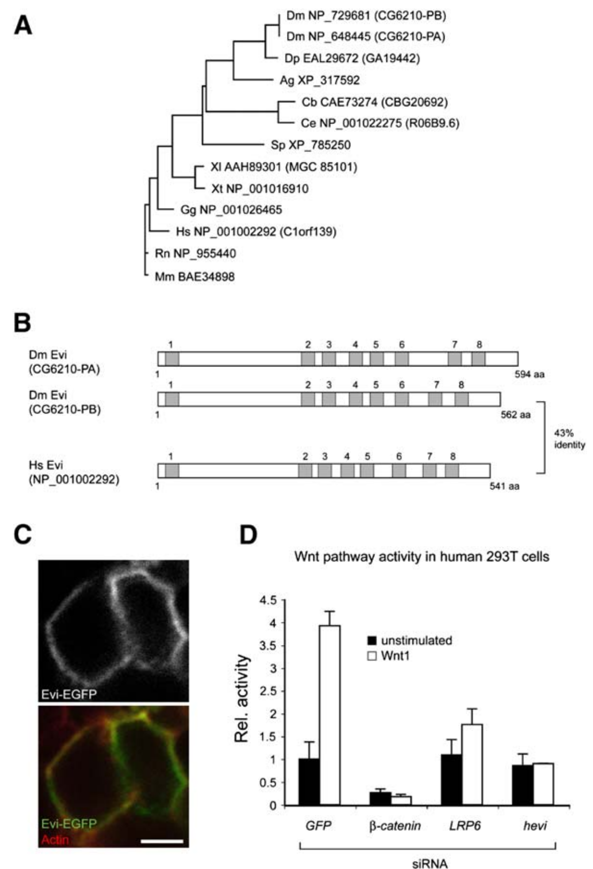

Figure 2. Evi Belongs to a Family of Conserved Multipass Transmembrane Proteins

(A) Phylogenetic tree of sequences from different species homologous to *Drosophila* Evi. GenBank accession numbers are indicated. Dm: *Drosophila melanogaster*; Dp: *Drosophila pseudoobscura*; Ag: *Anopheles gambiae*; Cb: *Caenorhabditis briggsae*; Ce: *Caenorhabditis elegans*; Sp: *Strongylocentrotus purpuratus*; Xl: *Xenopus laevis*; Xt: *Xenopus tropicalis*; Gg: *Gallus gallus*; Hs: *Homo sapiens*; Rn: *Rattus norvegicus*; Mm: *Mus musculus*.

(B) The predicted structures of both splice variants of the *Drosophila evi* gene (CG6210-PA and CG6210-PB) contain eight transmembrane domains (1–8). Note that the first transmembrane domain overlaps with a predicted signal peptide, possibly indicating a signal anchor. Sequence identity with the human homolog (accession number NP_001002292) is indicated.

(C) Evi-EGFP localizes to the plasma membrane of wing disc cells. Evi-EGFP (green) was expressed using a *dpp-gal4* driver. Shown is a confocal image; cells are outlined by staining for cortical actin with phalloidin-TRITC (red). Scale bar = 2 μm.

(D) RNAi in HEK293T cells against known positive Wnt regulators (β-*catenin* and *lrp6*) changes reporter induction relative to a negative control siRNA (*GFP*). siRNAs against the human homolog of *evi* (*hevi*) reduce mWnt1-induced reporter activity similar to β-*catenin* and *lrp6*. Relative activity is defined as mWnt1-activated firefly luciferase expression normalized against constitutively expressed *Renilla* luciferase. Data are represented as mean ± SD.

evi Is Required for Wg-Dependent Signaling In Vivo

During *Drosophila* development, Wg signaling is required for the patterning of embryonic and larval tissues. In the *Drosophila* embryo, Wg protein is secreted from cells in posterior compartments of parasegments and is required for proper cell-fate determination. Specified cells secrete and pattern cuticular structures, so-called denticle belts, which alternate with naked regions. In *wg* mutants, this alternating pattern is disturbed and naked cuticle cell fate is suppressed, leading to a characteristic lawn of denticles (van den Heuvel et al., 1989; Bejsovec and Martinez Arias, 1991). Later, during patterning of wing imaginal discs, Wg is secreted from a small stripe of cells at the dorsoventral boundary and signals to nearby cells that give rise to adult structures, such as the wing margin (Neumann and Cohen, 1997; reviewed in Cadigan, 2002).

To analyze *evi* function in vivo, we searched for insertions in the *evi* locus and identified the P element (EY01593) inserted in the 5′UTR (Bellen et al., 2004) (Figure 3A). Examination of the wings of *evi*^{EY01593} homozygous flies shows that the P element insertion is associated with wing defects. These include reduced wing size

and the loss of bristles at the posterior wing margin (Figure 3C). The phenotype is enhanced by the deficiency *Df(3L)lxd6*, which removes the *evi* locus (Figure 3D), and is reversible by precise excision of the P element (data not shown). This indicates that *evi*^{EY01593} phenotypes are due to the P element insertion in the *evi* locus.

In order to further analyze the biological role of *evi*, we screened for *evi* null alleles after remobilization of the *EY01593* P element. We recovered an imprecise excision, *evi*^{2}, which removes 771 bp of the *evi* gene, including 627 bp encoding the N-terminal 209 amino acids (Figure 3A). *evi*^{2} mutant flies are homozygous pupal lethal. We assume that *evi*^{2} is a complete loss-of-function allele, as its phenotype is not enhanced by crossing *evi*^{2} over *Df(3L)lxd6* (data not shown). A small number of zygotic *evi*^{2} mutants survive until late pupal stages (<2%), and those that do exhibit severe developmental defects such as the loss of leg structures and enlarged eyes indicative of Wg signaling defects (Campbell and Tomlinson, 1998; Baonza and Freeman, 2002) (Figure 4). The lethality and developmental phenotypes associated with the *evi*^{2} allele are rescued by expression of Evi-EGFP driven by the *actin-Gal4* driver. Overexpression of Evi-EGFP in a wild-type genetic background does not result in visible phenotypes (data not shown).

As in situ hybridization data on *CG6210/evi* show maternal expression (BDGP in situ database; Tomancak et al., 2002), we tested whether *evi*^{2} mutants survive to pupal stages due to maternal contribution. We generated germline clones using the FRT-FLP technique (Chou and Perrimon, 1996). As shown in Figure 3F, *evi*^{2} mutants devoid of both maternal and zygotic transcripts die during embryogenesis with a severe segment polarity phenotype that phenocopies mutations in core Wg pathway components (Baker, 1988; Bejsovec and Martinez Arias, 1991; Nusslein-Volhard and Wieschaus, 1980).

Since several components of Wnt/Fz pathways have been implicated both in the canonical and the planar cell polarity (PCP) pathway (reviewed in Klein and Mlodzik, 2005), we asked whether *evi*^{2} mutants have PCP defects. As shown in Figure 4, typical PCP phenotypes such as those caused by *dsh*^{1} could not be detected in *evi*^{2} pharate adults. Whereas bristles on *dsh*^{1} pharate adults were disorientated as typical for PCP phenotypes, nota bristles of *evi*^{2} mutants were aligned similar to wild-type flies. PCP defects were also not seen in *evi*^{2} mutant clones on wings (data not shown), suggesting that *evi* is specifically required for the canonical Wg pathway.

evi Is Required for Wg Target-Gene Expression during Wing Patterning

Since loss of *evi* causes developmental defects reminiscent of *wg* loss-of-function phenotypes, we tested

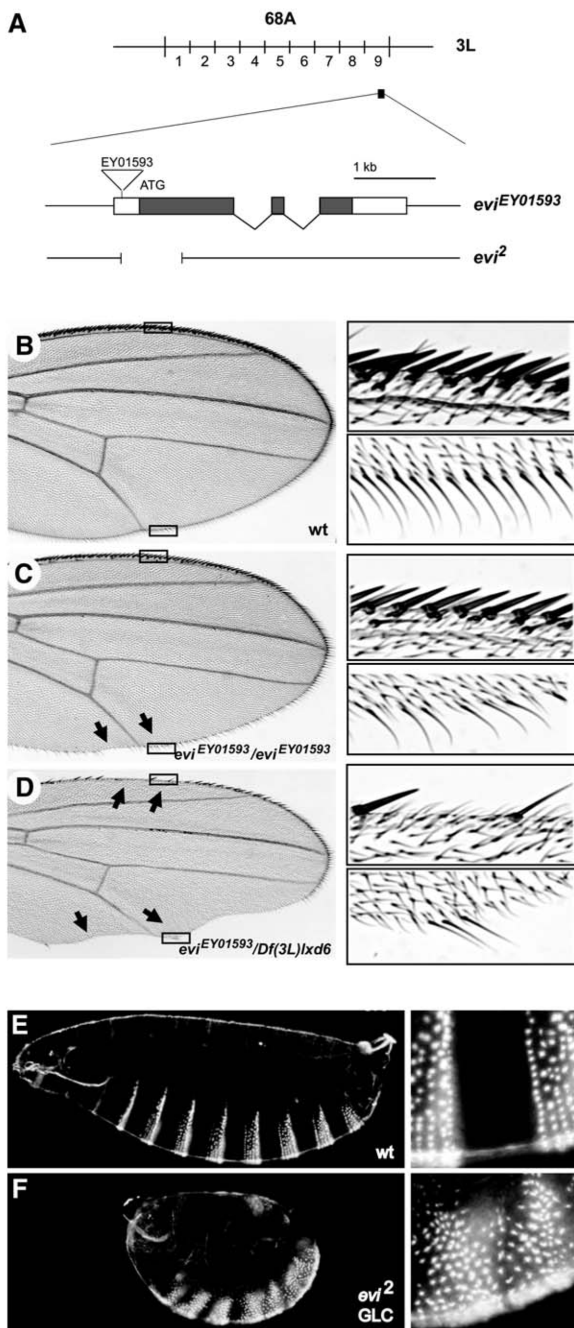

Figure 3. *evi* Loss-of-Function Mutants Show Segment Polarity and Wing-Margin Defects

(A) The *evi* locus maps to chromosome 3L at position 68A and encodes for a putative alternatively spliced transcript. The allele *evi*^{EY01593} contains a P element inserted in the 5′UTR of *evi*, 152 bp upstream of the first ATG. *evi*^{2} lacks 771 bp of the wild-type gene, including 627 bp encoding the first 209 amino acids.

(B–D) Wings of wild-type *Ore*^{R} (wt, [B]) flies and *evi* loss-of-function mutants. Right panels are enlargements of wing-margin regions outlined in the left panels. Wings of homozygous *evi*^{EY01593} flies (C) have a reduced number of posterior wing-margin bristles (arrows), and the evenness of the bristles' distribution is interrupted. The phenotype is enhanced by the deficiency *Df(3L)lxd6* deleting the *evi* gene region, including partial loss of posterior wing margin and reduced number of anterior wing-margin bristles ([D], arrows).

(E and F) Cuticle preparations of *Ore*^{R} (wt, [E]) and *evi*^{2} germline clone embryos (*evi*^{2} GLC, [F]). In wild-type cuticles, denticle belts alternate with naked regions. *evi*^{2} cuticles are smaller and lack naked regions, resembling the *wg* segment polarity phenotype.

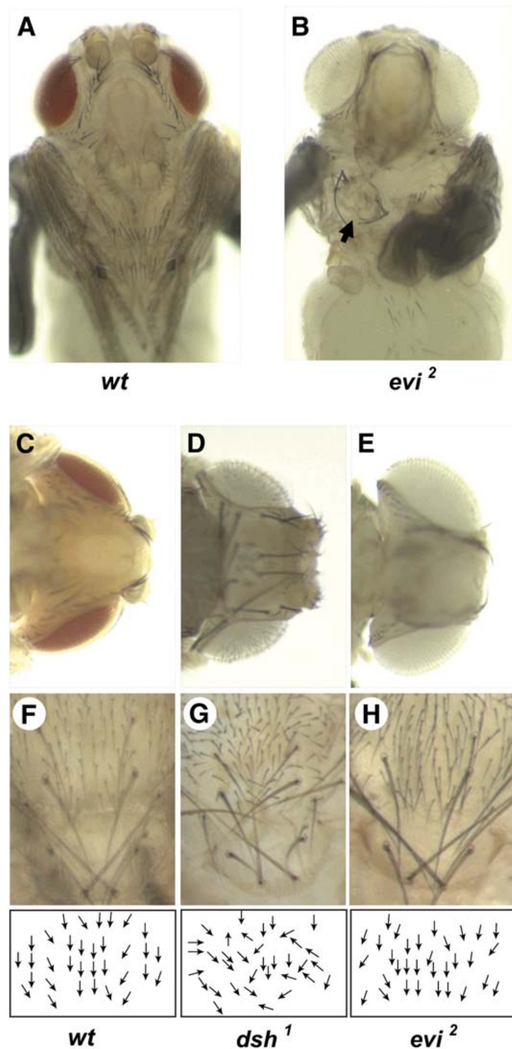

Figure 4. *evi* Is Involved in Canonical Wg Signaling and Is Not Required for Planar Cell Polarity

Pharate adults prepared from late pupae. Ore^R (wt) morphology is shown in (A), (C), and (F).

(A–E) evi^2 pharate adults show severe developmental defects such as loss of legs ([B], arrow) compared to wt (A) and oversized eyes (E) compared to wt (C) and dsh^1 (D).

(F–H) Nota of wt, dsh^1, and evi^2 pharate adults. While the orientation of bristles on dsh^1 mutants is disturbed, bristles on nota of wild-type and evi^2 mutant animals are aligned along the anterior-posterior axis. The directions of bristles are schematically represented by arrows in the lower panels.

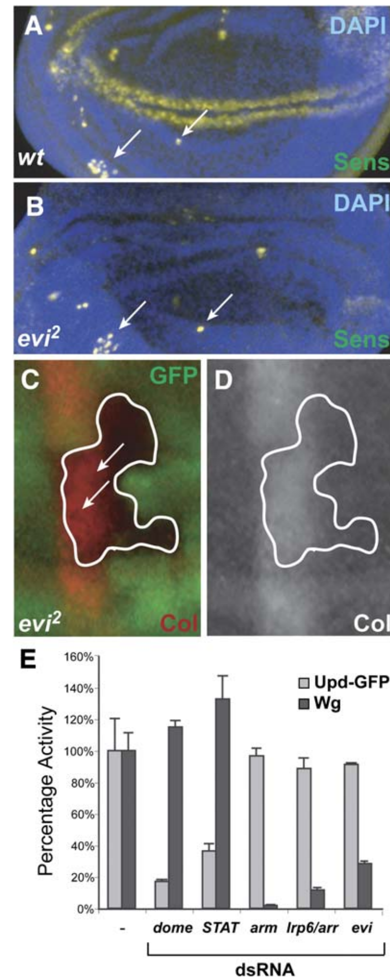

Figure 5. Specific Requirement of *evi* for Wg Target-Gene Expression

(A and B) Wing discs stained for Sens protein (green; yellow in overlay with DAPI) and DNA (blue). Sens is expressed in wild-type cells in two narrow stripes of cells along the dorsoventral boundary in a Wg-dependent manner and in neuronal clusters independently of Wg (A). Wg-dependent Sens expression is absent in evi^2 mutant discs, while it is still present in neuronal clusters ([B], arrows).

(C and D) Hh target-gene expression is not affected in evi^2 mutant clones. Clones are marked by the absence of GFP (green) and are outlined; Col is red. Shown is a merge of the green and red channel (C) and the red channel alone (D). Col is expressed in a stripe of anterior cells along the A/P boundary, while Hh is secreted by posterior cells (anterior: right; posterior: left). Col expression remains unchanged in the evi^2 mutant clone compared to neighboring wild-type tissue.

(E) *evi* is required for Wg signaling, but not for JAK/STAT signaling, in $S2R^+$ cells. JAK/STAT- or Wg-signaling activity was measured in $S2R^+$ cells using an Upd-GFP- or Wg-responsive luciferase reporter assay. dsRNAs against known JAK/STAT components (*dome* and *stat*) reduce JAK/STAT but not Wg reporter activity. dsRNAs against *arm*, *lrp6/arr*, and *evi* reduce Wg but not JAK/STAT reporter activity. Data are represented as mean ± SD.

whether Wg target-gene expression is affected in *evi* mutant animals. The Wg target gene *senseless* (*sens*) is expressed at the dorsoventral boundary, where its expression depends on high levels of Wg, as well as in neuronal clusters on the notum anlage, independent of Wg (Nolo et al., 2000). We observed that Wg-dependent expression of *sens* was abolished in evi^2 mutant wing discs, whereas it was still expressed in the Wg-independent neuronal clusters (Figure 5B).

In order to test the specificity of *evi* for Wg signaling, we stained wing discs containing evi^2 mutant clones for the expression of the Hh target gene *collier* (*col*). *col* is

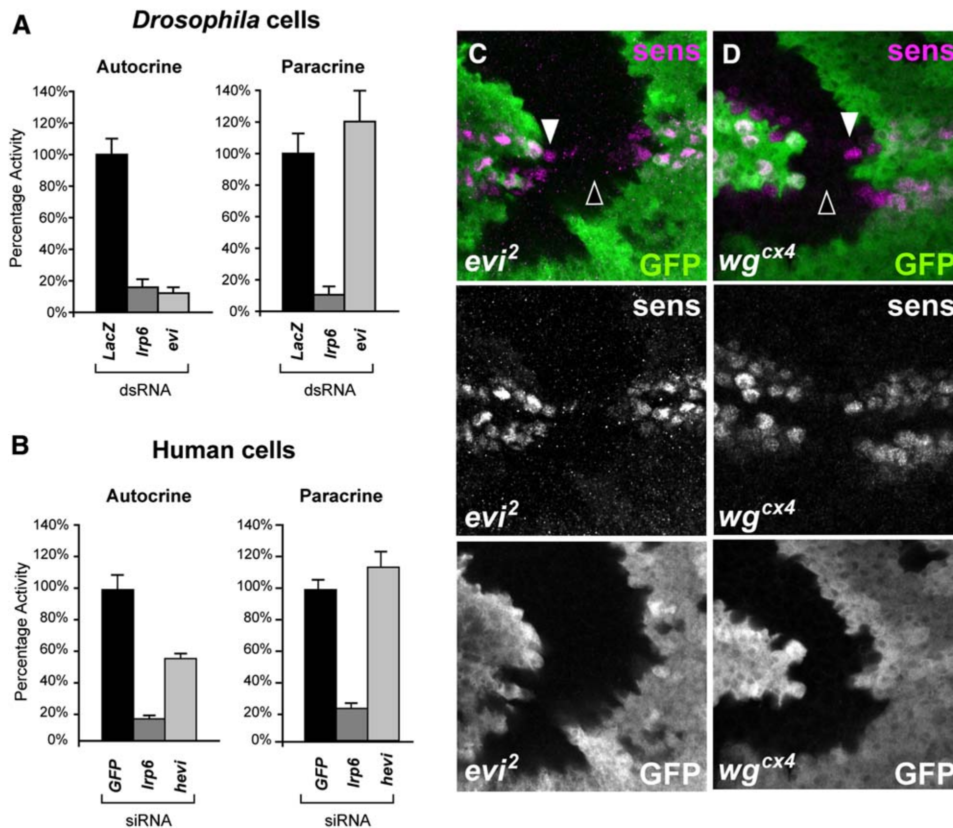

Figure 6. *evi* Acts in the Wnt/Wg-Secreting Cell in *Drosophila* and Human Cells

(A) *Drosophila* S2R+ cells transfected with a Wg reporter construct were either cotransfected with *pAc-wg* for autocrine activation (left panel) or mixed with S2 GFP-Wg cells for paracrine activation (right panel). RNAi was induced only in reporter-transfected cells by incubation with dsRNAs against *lacZ*, *lrp6/arr*, or *evi*. RNAi against *evi* reduces reporter activity in cells that express Wg in an autocrine manner, but not in cells that receive external GFP-Wg. dsRNA against *lrp6/arr* affects pathway activity in both cases, whereas dsRNA against *lacZ* has no effect.

(B) HEK293T cells transfected with the Wnt reporter construct were either cotransfected with *pCS2-mWnt3a* for autocrine activation (left panel) or incubated in *mWnt3a*-conditioned medium for paracrine activation (right panel). RNAi against *hevi* reduces reporter activity in cells that express mWnt3a, but not in cells that receive mWnt3a-conditioned medium. siRNA-mediated knockdown of *lrp6* reduces reporter activity in both cases, whereas siRNA against GFP has no effect. Data are represented as mean ± SD.

(C) Sens expression in evi^2 mutant clones on wing discs shows cell nonautonomy of *evi*. Clones are marked by the absence of GFP. The Wg target Sens (pink), which needs high amounts of Wg, is expressed in mutant cells at the clone border (filled arrowhead), but not in mutant cells in the middle of the clone (open arrowhead). These cells (filled arrowhead) receive Wg from wild-type neighboring cells.

(D) Nonautonomous rescue of Sens expression is also observed in wg^{cx4} mutant clones.

expressed in anterior compartment cells adjacent to the A/P boundary (Vervoort et al., 1999). In anterior evi^2 clones at the A/P boundary (Figure 5) and in evi^2 mutant discs (data not shown), *col* was still expressed, indicating that *evi* is required for Wg- but not Hh-target-gene expression. We also tested the effect of *evi* loss of function on JAK/STAT signaling. In induced cultured *Drosophila* cells responsive to JAK/STAT signaling, we depleted *evi* as well as two known regulators of JAK/STAT signaling, *domeless* and *STAT92E* (Muller et al., 2005), by RNAi. As shown in Figure 5E, knockdown of *evi* did not affect reporter activity, while RNAi against *domeless* or *STAT92E* reduced the pathway activity. Together with the in vivo phenotypes, this supports a model whereby *evi* is required not for multiple pathways but specifically for Wg signaling.

evi Is Required in the Wg-Producing Cell

We next set out to map where in the Wg pathway *evi* acts. In *Drosophila* cells, we activated Wg signaling by expression of Wg, Dsh, or LRP6ΔN, a constitutively active form of hLRP6. These factors were previously shown to activate the Wg pathway (Cong et al., 2004; Yanagawa et al., 1995). When we induced RNAi against *evi*, reporter activity was reduced in cells activated with Wg, but not in cells activated with LRP6ΔN or Dsh (Figure S3). In contrast, RNAi against *arm,* a pathway regulator acting downstream of *dsh*, resulted in reduced Wg reporter activity in all three cases. We therefore conclude that *evi* acts upstream of *lrp6/arr* and *dsh* in Wg signaling.

We then tested whether *evi* regulates Wg signaling in the Wg-secreting or -receiving cell. We transfected the

reporter constructs, depleted *evi* by RNAi, and activated Wg signaling in either an autocrine manner by cotransfection of *pAc-wg* or a paracrine manner by mixing the reporter-transfected cells with S2 cells that stably express GFP-Wg (Piddini et al., 2005). RNAi targeting *evi* reduced reporter activity in cells that were activated in an autocrine manner, but not in cells that received exogenous GFP-Wg. *lrp6/arr* dsRNA caused the reduction of reporter levels in both cell populations, suggesting that, unlike *lrp6/arr*, *evi* is required in the Wg-producing cell (Figure 6A).

To test whether the *evi* human homolog *hevi* is also required in the ligand-secreting cell, *hevi* or *lrp6* was depleted in HEK293T cells by RNAi, and Wnt signaling was activated by either cotransfection of the reporter constructs with an expression plasmid for mouse Wnt3a (autocrine) or incubation of the reporter-transfected cells in mouse Wnt3a-conditioned medium (paracrine). As shown in Figure 6B, knockdown of *hevi* by RNAi reduced reporter activity in cells that were activated in an autocrine manner, but not in cells that were treated with Wnt3a-conditioned medium. *lrp6* knockdown led to the reduction of reporter levels in both cases, suggesting that, as in *Drosophila*, *hevi* is required in Wnt-producing cells in mammals.

We then analyzed wing imaginal discs containing evi^2 mutant clones for Wg target-gene expression. As shown in Figure 6C, Sens was detected in *evi* mutant cells close to the clonal border, whereas Sens expression was diminished in cells further away (see also Figure S4). This phenotype is similar to that of *wg* mutant clones (Figure 6D and Figure S4), indicating that *evi* acts in a cell-nonautonomous manner. Moreover, the analysis of *dll*, a Wg target gene that requires only low levels of signaling activity, was rescued in clones by surrounding wild-type tissue; clonal analyses of wg^{cx4} mutant alleles show the same phenotype (Figure S5). *evi* clones that do not overlap with the dorsoventral boundary express target genes normally, consistent with a model whereby *evi* is specifically required in Wg-producing cells.

To test whether *evi* loss of function would affect the expression or distribution of Wg, we stained wing imaginal discs for Wg protein (Figure 7 and Figure S6). In order to detect total Wg, we used a staining protocol that involves permeabilization of the cells prior to incubation with the anti-Wg antibody (referred to as Wg (total)). In evi^2 mutant clones, the intensity of the Wg signal is stronger than in adjacent wild-type tissue, suggesting that, in the absence of *evi*, Wg was still expressed but was retained in secreting cells (Figure 7 and Figure S7). Accumulation of Wg was also seen in evi^2 mutant embryos (Figure S6), suggesting that *evi* acts in Wg-producing cells. The phenotype is specific for Wg as Hh distribution was not changed in evi^2 mutant clones (Figure S8).

To test whether Wg accumulates in evi^2 mutant cells, we stained wing imaginal discs containing evi^2 mutant clones for extracellular Wg (Strigini and Cohen, 2000). As shown in Figure S7, extracellular Wg was reduced in large evi^2 mutant clones, suggesting that Wg-expressing cells fail to secrete Wg.

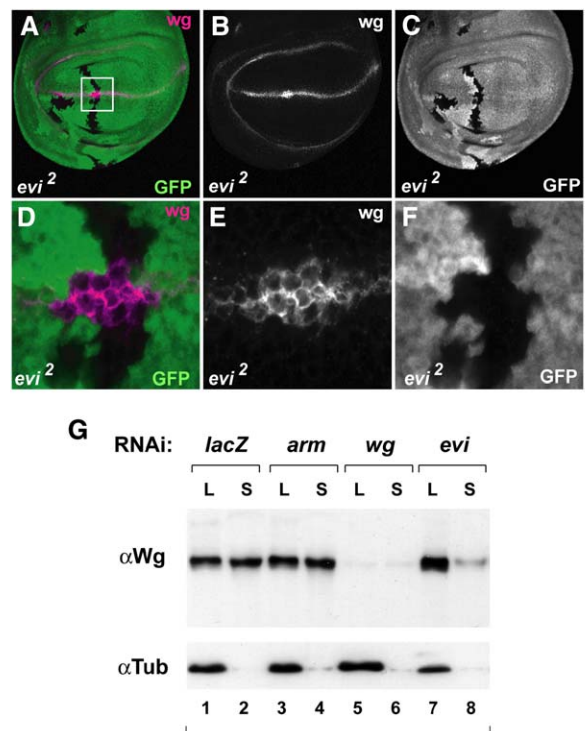

Figure 7. *evi* Is Required for Wg Secretion

(A–F) Third-instar wing imaginal discs with evi^2 clones (marked by the absence of GFP) stained for total Wg with an anti-Wg antibody (pink). (D)–(F) show the enlarged region outlined in (A) of the Wg channel (B and E), the GFP channel (C and F), and the merge (A and D).

(G) Western blot analysis of secreted GFP-Wg in cell culture media. The Western blot was probed with the anti-Wg antibody (αWg) and reprobed with an anti-β-tubulin (αTub) antibody to ensure equal amounts of starting material. Wg is present in both lysates (L) and supernatants (S) of cells treated with dsRNA against *lacZ* or *arm* (lanes 1, 2, 3, and 4), but is significantly reduced in the supernatant of cells treated with *evi* dsRNA (lane 8). dsRNA against *wg* was used as a positive control (lanes 5 and 6).

To confirm that *evi* is required for Wg secretion, we assessed amounts of GFP-Wg in the media of stably transfected cells depleted for *lacZ*, *arm*, *wg*, or *evi*. As shown in Figure 7G, GFP-Wg was reduced in supernatants of cells treated with *wg* or *evi* dsRNA, whereas cells treated with negative control dsRNAs secrete Wg normally. Together, these results indicate that Wg is retained in the absence of *evi*.

As Wg is localized at the apical side of cells at the dorsoventral border in wild-type wing discs (Strigini and Cohen, 2000), we asked whether Wg would accumulate apically at the plasma membrane of evi^2 mutant cells. We therefore analyzed Wg localization in evi^2 mutant wing discs. Whereas Wg was detected apically in wild-type cells, Wg was found throughout evi^2 cells (Figure S6B), suggesting that *evi* might be required for the proper subcellular distribution of Wg.

DISCUSSION

How organisms control the spatial and temporal activation of signaling pathways remains an important question in biology. With the availability of whole genome sequences, one of the key challenges is the functional annotation of the encoded gene products. RNAi approaches have become a powerful genetic tool to systematically dissect cellular pathways (Boutros et al., 2004; DasGupta et al., 2005; Muller et al., 2005). Here, we have analyzed the consequences of depleting almost all known *Drosophila* transmembrane proteins for Wg signaling. The survey in cultured cells identified Fz, Fz4, and LRP6/Arr, transmembrane proteins known to positively regulate Wg signaling. It is interesting to note that both Fz and Fz4, but not Fz2, appear to be required in cells for Wg signal transduction in a nonredundant manner. Moreover, we have identified a previously uncharacterized locus, *evi/CG6210*, which is a member of an evolutionarily conserved gene family, as a novel regulator of Wnt/Wg signaling both during *Drosophila* development and in human cells. *evi* has not been found in other RNAi screens for Wg signaling components (DasGupta et al., 2005). In imaginal discs and embryos, we show that *evi* is specifically required for Wg secretion and that loss of *evi* leads to accumulation of Wg in the producing cell.

While significant progress has been made in our understanding of how signals are transduced from the ligand-activated receptor to the nucleus in the signal-receiving cell, little is known about the events that lead to the secretion of a functional ligand from the producing cell. Recent studies investigating Hh signaling have identified several factors required in secreting cells, which posttranslationally modify the ligand prior to secretion (reviewed in Mann and Beachy, 2004).

Like Hh, Wg has been demonstrated to be lipid modified, a modification that accounts for its hydrophobicity and its targeting to lipid rafts. This posttranslational modification is probably mediated by the ER-resident protein Porcupine (Por) (Tanaka et al., 2002; Zhai et al., 2004). To date, Por remains the only factor known to be specifically required for Wg secretion. Another factor implicated in the secretion of Wg is the dynamin Shibire (Shi) (Strigini and Cohen, 2000). However, Shi has been shown to have a general function in vesicular transport rather than to be dedicated exclusively to Wg secretion (Jones et al., 1998; Moline et al., 1999; Strigini and Cohen, 2000).

Here, we show that the multipass transmembrane protein Evi is specifically required for Wg/Wnt secretion across species. Three independent lines of evidence suggest that Evi has a specific role in Wnt signaling. First, loss-of-function alleles phenocopy *wg* mutations and do not show other obvious phenotypes. During early embryonic development, germline clones of *evi* phenocopy mutations in other core Wg pathway components. The phenotype of *evi* is similar to *wg* during wing imaginal-disc development. Second, *evi* knockdown does not prevent the JAK/STAT ligand Upd from activating a cell-based reporter. Third, in vivo analysis demonstrates that *evi* loss of function leads to neither abrogation of Hh target-gene expression in receiving cells nor Hh accumulation in producing cells. This demonstrates that Evi is not required for the secretion of either Upd or Hh ligands and supports the model that Evi is not part of a general secretory machinery. Interestingly, Evi does not seem to be involved in the PCP signaling pathway, which might indicate that Fz-dependent PCP signaling in *Drosophila* might not rely on a Wnt factor.

While there is no evidence that *evi* acts in other signaling pathways, our results indicate that *evi* is a core Wg/Wnt signaling-pathway component in both invertebrates and vertebrates. Cell-based experiments in *Drosophila* and human cells show that Wg/Wnt signaling is impaired when *evi* is depleted by RNAi. In vivo, germline and somatic clones of *evi* phenocopy *wg* loss-of-function mutations.

Our results show that Evi is necessary for efficient secretion of Wg. Cell culture experiments and clonal analyses in imaginal-disc tissues demonstrate that *evi* is required in the Wg-producing cell. Like *wg*, *evi* shows cell-nonautonomous phenotypes on target-gene expression in somatic cell clones. We observed that Wg accumulates in Wg-producing cells in an *evi* mutant background. We also show that Wg is not efficiently released into the supernatant of cultured cells treated with *evi* dsRNA. In addition to the embryonic phenotype, these results demonstrate an impaired Wg secretion when *evi* is absent. However, target genes that require only low levels of Wg, such as *dll*, are expressed in evi^2 mutant discs. This phenotype can be explained by maternally contributed *evi* transcript, although it cannot be excluded that Wg is still secreted in low amounts by *evi* mutant cells.

Sequence analysis of *evi* predicts that the gene encodes a transmembrane protein. Consistently, an Evi-EGFP fusion protein localizes to the plasma membrane (Figure 2C and Figure S1), suggesting that Evi, unlike the ER-resident protein Por, might regulate Wg secretion at the plasma membrane. Sequence analysis does not indicate any obvious enzymatic activity, such as glycosyl- or acyltransferase activity. However, we show that in polarized cells, such as wing imaginal-disc cells, loss of *evi* leads to the delocalization of Wg protein from the apical side. In mammalian cells, apical secretion of proteins has been attributed to specialized secretory pathways that include protein sorting into cholesterol/sphingolipid-rich microdomains (lipid rafts) within the *trans*-Golgi network (reviewed in Ikonen and Simons, 1998). It has been shown that acylation of Wg by Por is required for incorporation of Wg in polarized vesicles that transport Wg to specific sites at the plasma membrane (Zhai et al., 2004). Since loss of *evi* leads to the delocalization of Wg protein from the apical side, we propose that Evi might be involved in the proper localization of Wg prior to its secretion. However, in *evi* mutant cells, accumulation of Wg alone may account for its delocalization and may not be due to a specific involvement of Evi in apical sorting.

Interestingly, apical localization of Wg-containing vesicles has been shown to be involved in Wg signaling in the *Drosophila* embryo (Pfeiffer et al., 2002). A possible function of Evi may lie in the packaging, localization, or fusion of such Wg-containing vesicles.

We show here that Evi, a conserved transmembrane protein, is required for efficient secretion of Wg in embryos and from imaginal-disc cells in vivo. As Evi appears to localize to the plasma membrane, it is tempting to speculate that the biological role of Evi is downstream of the ER-resident protein Por in the secretion of Wnts. Our results support a model in which the cellular release of growth factors is performed by dedicated machineries that provide an additional level of regulation for the secretion of ligands. Evi might therefore belong to a growing family of specialized factors that regulate the cellular release of specific families of growth factors.

EXPERIMENTAL PROCEDURES

Cell Culture

Drosophila Kc_{167}, S2R+, and S2 GFP-Wg cells (Piddini et al., 2005) were maintained at 25°C in Schneider's medium (Invitrogen) supplemented with 10% fetal calf serum (PAA) and 50 μg/ml penicillin/streptomycin (Invitrogen). HEK293T cells were maintained in Dulbecco's MEM (GIBCO) supplemented with 10% fetal calf serum (PAA), 50 μg/ml penicillin/streptomycin (Invitrogen), and 2 mM L-glutamine at 37°C in a humidified atmosphere with 5% CO_2. *Drosophila* cells were transfected in 6- or 24-well plates with 2 μg or 0.5 μg of total DNA, respectively, using Effectene (QIAGEN). Human cells were transfected with 0.1 μg of DNA or 100 nM siRNAs in 96-well plates using FuGene (Roche) or Oligofectamine (Invitrogen).

Plasmid Constructs

pRp128-RL was generated by cloning the promoter region (−250 to +6) of the *Drosophila* RNA polymerase III 128 kDa subunit into *pRL null* (Promega). *pAc-wg* and *pAc-lrp6ΔN* were constructed by inserting the *wg* cDNA (Stapleton et al., 2002 and *Drosophila* Genomics Resource Center) or *lrp6ΔN* (Cong et al., 2004) into *pAc5.1/V5-His* (Invitrogen). The *evi* ORF lacking the stop codon was amplified from an *evi* full-length cDNA (GH01813, *Drosophila* Genomics Resource Center) and cloned into *pEGFP-N1* (Clontech). *evi-EGFP* was then subcloned into *pAc5.1/V5-His* (Invitrogen) and *pUAST* (Brand and Perrimon, 1993). *pActin-Upd-GFP* and *2x6xDraf-Luciferase* have been previously described (Muller et al., 2005).

Sequence Analysis

Transmembrane proteins to be screened were selected by batch analysis of open reading frames using the TmHmm program. Evi homologous sequences were retrieved from GenBank (http://www.ncbi.nlm.nih.gov/) using BLAST. Sequences were aligned using the ClustalW program, and the phylogenetic tree of aligned sequences was constructed using TreeView X (Page, 1996). The Evi predicted protein sequence was analyzed for transmembrane regions using SMART (Letunic et al., 2006). Note that the first predicted transmembrane domain overlaps with a predicted signal peptide, suggesting a signal anchor.

RNAi Experiments

For RNAi experiments in *Drosophila* cells, dsRNAs were generated from DNA templates (Hild et al., 2003) by in vitro transcription as described previously (Boutros et al., 2004). Complete sequence information of the dsRNAs contained in the library is available at http://rnai.dkfz.de/. RNAi screening was performed as previously reported (Muller et al., 2005). For RNAi experiments, 5×10^6 Kc_{167} cells/well were transfected in batch in 6-well plates with 0.5 μg of the *pLef-FL* Wg-responsive reporter (Giese et al., 1995), 0.5 μg of *pActin-Lef-HA* expression plasmid, 0.5 μg of *pAc-wg* for pathway induction, 0.25 μg of the *pRp128-RL* coreporter, and 0.25 μg of *pAc5.1-Sid-1* to enhance dsRNA uptake (Feinberg and Hunter, 2003). Twelve hours after transfection, cells were resuspended in serum-free medium to a concentration of 7.5×10^5 cells/ml, and 20 μl/well was transferred to the 384-well screening plates preloaded with 0.5 μg of dsRNA in 5 μl of 1 mM Tris (pH 7.0) using an automated liquid dispenser (Multidrop, Thermo Labsystems). The cells were incubated at 25°C for 1 hr; subsequently, 30 μl of serum-containing medium was added to each well, and cells were grown under normal cell culture conditions for 5 days to allow for protein depletion. Each RNAi experiment was performed in duplicate. Firefly and *Renilla* luciferase activity was measured on a Mithras LB940 plate reader (Berthold Technologies). dsRNAs with strong viability phenotypes or variability between replicates were excluded from further analysis. Quantile-quantile plots show normally distributed data against z scores of actual pathway screening results of putative transmembrane proteins. Data analysis and representation were performed using Bioconductor/R as described previously (Muller et al., 2005). dsRNAs targeting the 3′UTR of *evi* were generated using primers 5′-TAATACGACTCACTATAGGGATTCTGTGCATTCGACAACG-3′ and 5′-TAATACGACTCACTATAGGGTCGACAATTTGGCACTTGAAAC-3′.

For the paracrine signaling assay, cells transfected with the reporter constructs were mixed with S2 GFP-Wg cells (Piddini et al., 2005) at a ratio of 1:3 48 hr after dsRNA treatment. Luciferase assays were performed 5 days after dsRNA treatment. Retesting experiments were performed in quadruplicate (without transfection of the *Sid-1*).

For the epistasis analysis in *Drosophila* cells, $S2R^+$ cells were transfected with reporter plasmids in 6-well plates as described above and, for pathway induction, with 0.5 μg of *pAc-wg*, *pAc-lrp6ΔN*, or *pMT-dsh* (Yanagawa et al., 1995). Twelve hours after transfection, cells were resuspended in serum-free medium at a concentration of 7.5×10^5 cells/ml, and 20 μl/well was transferred to the 384-well assay plates containing 0.3 μg of dsRNA. The cells were incubated at 25°C for 1 hr, and 30 μl of serum-containing medium was added to each well. Dsh expression was induced with 500 μM $CuSO_4$ solution 48 hr after dsRNA treatment.

For RNAi experiments in human cells, siRNAs against β-*catenin* (M-003482-00-0005), *lrp6* (M-003845-01-0005), *hevi* (M-018728-00-0005), or *GFP* control targets (K-002800-C3-01) were obtained from Dharmacon. For RNAi in HEK293T cells, cells were transfected in 96-well plates with 100 nM siRNA using Oligofectamine (Invitrogen). Forty-eight hours later, cells were transfected with TopFlash (van de Wetering et al., 1997), *pCMV-RL*, *pCS2-mFz8*, *pCS2-LRP6*, *pCS2-mWnt1*, *pCS2-Wnt3a*, or empty vector using Fugene (Roche). Cells were then incubated under normal cell culture conditions in DMEM/10% FCS for 24 hr. For paracrine activation, Wnt3a-conditioned medium (a kind gift from C. Niehrs) was added to the cells 12 hr after plasmid transfection. Luciferase assays were performed 24 hr after plasmid transfection.

Genetics

The P element line *P{EPgy2}MRP*^{EY01593} (Bellen et al., 2004) and the deficiency *Df(3L)lxd6* were obtained from the Bloomington Stock Center. (Note that CG6210 was misannotated in Flybase as MRP1, which is encoded by the CG6214 locus.) The EP element was mobilized using a *y,w;;Sb, Δ2–3/TM6, Ubx* strain as a source of transposase. The white-eyed *TM3 SbSer* males (excision events) were individually crossed with a *TM3 SbSer/Dr* strain to establish stocks. *evi*^{2} is a 771 bp deletion covering the start codon. For the generation of *evi* mutant clones, *evi*^{2} was recombined on an FRT80 chromosome, and isogenised stocks were established using a *TM3 SbSer/Dr* strain. *FRT80 evi*^{2}*/TM3 Sb* males were then crossed with *y,w, hs-flp;;FRT80 Ub-GFP* virgins. For the generation of *wg* clones, *FRT40 wg*^{cx4}*/CyO* males were crossed with *y,w, hs-flp;FRT40 Ub-GFP* virgins. Several heat shocks

were performed on the progeny in 24 hr intervals, for 1.5 hr at 37°C. Transgenic flies carrying *pUAST-evi-EGFP* were obtained using standard techniques.

For the generation of germline clones, *FRT80 evi²/TM3 Sb* females were crossed with *w, hs-flp slbo lacZ; FRT80 P[ovoD]/TM3Ser* males (a kind gift from H. Sung and P. Rorth). The progeny were exposed to several heat shocks during the second and third larval stage with 24 hr interval to induce recombination. Mosaic virgins were then crossed with *evi²/TM6b* males, and embryos were collected on agar plates.

Clonal Analysis and Immunostainings

For target-gene analysis, wing discs were dissected from third-instar larvae, fixed in 4% paraformaldehyde/PBS for 20 min at room temperature, washed 3 times with PBS, permeabilized for 45 min in 0.2% Triton/PBS, blocked in 1% BSA/PBS for 1 hr, and incubated with a guinea pig anti-Sens antibody (Nolo et al., 2000) at a dilution of 1:1000 or a rat anti-Dll antibody (gift from S. Cohen) at a dilution of 1:500, together with a rabbit anti-GFP antibody at a dilution of 1:1000 at 4°C overnight. Secondary detection was performed using standard fluorescently labeled antibodies and protocols (Dianova). Extracellular and total Wg stainings were performed as described in Strigini and Cohen (2000). A Zeiss Axiovert 200M microscope or a Leica DM IRE2 confocal microscope was used for microscopy analysis. Images were assembled and processed in Adobe Photoshop and ImageJ.

Embryos were dechorionated in 50% bleach for 2–3 min, rinsed, and fixed for 30 min in 50% heptane, 50% fixative (37% formaldehyde [Sigma] in PBS). After fixation, embryos were devitellinized by incubation in methanol and shaking. After four washes with methanol, embryos were rehydrated first in 50% MeOH/PBS and then in PBS. After blocking for 2 hr at 4°C, embryos were incubated overnight at 4°C with a mouse anti-Wg antibody at a dilution of 1:100. Secondary detection was performed using standard fluorescently labeled antibodies and protocols (Dianova). A total of 25 embryos were analyzed by microscopy.

Wg Secretion Assay

2×10^6 S2 GFP-Wg cells were seeded on 20 μg of dsRNA in 1 ml serum-free media in 6-well plates and incubated at 25°C for 1 hr before 1 ml of serum-containing medium was added. After 2 days, 50 μg/ml heparin was added to detach cell bound Wg. After 24 hr, the medium was replaced by medium containing 5% fetal calf serum and 375 μM $CuSO_4$ to induce GFP-Wg expression. Ten micrograms of dsRNA was added to each well to ensure continuous depletion of proteins. Twenty-four hours after medium exchange, heparin was added, and supernatants were collected 24 hr afterwards. Supernatants were centrifuged at 4,000 rpm to remove cells and subsequently at 12,000 rpm to remove any insoluble material. Cells were lysed in 100 μl of lysis buffer (0.1% Triton in PBS with protease inhibitor [Roche]) and centrifuged to remove cell debris. Protein concentrations were measured by Bradford assay (Bio-Rad). Ten micrograms of proteins from lysates and supernatants was separated by SDS-PAGE. Western blots were performed with standard methods using ECL plus detection (Amersham). Mouse anti-Wg and mouse anti-β-tubulin (a gift from U. Euteneuer) were used at a dilution of 1:1000.

Supplemental Data

Supplemental Data include Supplemental References and nine figures and can be found with this article online at http://www.cell.com/cgi/content/full/125/3/523/DC1/.

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