Sprinter: a novel transmembrane protein required for Wg secretion and signaling

Robyn M. Goodman¹, Shreya Thombre¹, Zeynep Firtina¹, Dione Gray¹, Daniella Betts¹, Jamie Roebuck², Eric P. Spana² and Erica M. Selva¹,*

Wingless (Wg) is a secreted ligand that differentially activates gene expression in target tissues. It belongs to the Wnt family of secreted signaling molecules that regulate cell-to-cell interactions during development. Activation of Wg targets is dependent on the ligand concentration in the extracellular milieu; cellular mechanisms that govern the synthesis, delivery and receipt of Wg are elaborate and complex. We have identified sprinter (srt), which encodes a novel, evolutionarily conserved transmembrane protein required for the transmission of the Wg signal. Mutations in srt cause the accumulation of Wg in cells that express it, and retention of the ligand prevents activation of its target genes in signal-receiving cells. In the absence of Srt activity, levels of Wg targets (including Engrailed in embryos lacking maternal and zygotic srt, and Senseless and Achaete in wing discs) are reduced. Activation of Wg targets in the receiving cells does not require srt. Hence, the function of Srt is restricted to events occurring within the Wg-producing cells. We show that srt is not required for any aspect of Hedgehog (Hh) signal transduction, suggesting specificity of srt for the Wg pathway. We propose that srt encodes a protein required for Wg secretion that regulates maturation, membrane targeting or delivery of Wg. Loss of srt function in turn diminishes Wg-pathway activation in receiving cells.

KEY WORDS: Wingless signaling, Wingless, Drosophila, Chaperone, Secretion

INTRODUCTION

Normal growth and development of multicellular organisms depends upon the spatially and temporally controlled reception of numerous extracellular signals that activate signal-transduction cascades within target cells. Whereas the signal-transduction pathways downstream from plasma-membrane receptors have been characterized, the complexity of the regulatory mechanisms that operate at the level of ligand processing in cells sending the signal, and the movement of ligands in the extracellular space, have only recently become apparent. Both of these aspects of ligand production are crucial for appropriate developmental signaling. Wingless (Wg), a secreted ligand responsible for differential gene expression in Drosophila, signals through a pathway initiated by the binding of Wg to its cognate seven-pass transmembrane receptor, which is encoded by the Frizzled family, and a low-density lipoprotein-like co-receptor (Arrow), which is highly conserved from flies to humans (reviewed in Giles et al., 2003). Only recently has it become clear that specialized mechanisms are required for the processing and packaging of the Wg ligand, the dissemination of the packaged ligand and its targeting to receiving cells for efficient Wg signal transduction.

A number of factors have been identified that are essential for efficient Wg signal transmission, but do not participate directly in the intracellular signaling pathway in cells receiving the signal. In porcupine (porc)-mutant embryos, Wg fails to be secreted, but instead accumulates inside Wg-producing cells. The failure to secrete Wg leads to a ‘lawn of denticles’ – an embryonic phenotype typical of Wg loss-of-function mutants (Kadowaki et al., 1996; Manoukian et al., 1995). Wnt proteins have recently been shown to be palmitoylated, and porc probably encodes a palmitoyltransferase necessary for this post-translational lipid modification (Willert et al., 2003; Zhai et al., 2004). In the porc mutant larvae, Wg is not lipid-modified and fails to be targeted to plasma-membrane lipid rafts (Zhai et al., 2004). The importance of rafts in cellular signaling is inferred from the observation that they are fortified with membrane molecules responsible for signal transduction (reviewed in Kurzchalia and Parton, 1999). Indeed, rafts might serve as an ideal platform for the accumulation and release of hydrophobic ligands, such as Wg and Hedgehog (Hh, both palmitoylated and cholesterol-modified), from the plasma membrane (Chamoun et al., 2001; Lee and Treisman, 2001; Micchelli et al., 2002; Porter et al., 1996).

Recent studies in Drosophila demonstrate that this is clearly the case, as active forms of secreted Wg and Hh are packaged into lipoprotein particles (Panáková et al., 2005). In larvae, lipophorin-enriched particles are probably synthesized in the fat body and come into contact with signaling cells, where they are loaded with their lipophilic cargo of modified Wg and modified Hh for dissemination to receiving cells (Panáková et al., 2005). It is now apparent that the correct processing of Wnts as they move through the secretory pathway, accurate membrane targeting and packaging at the plasma membrane are intricate and complex processes. Failure of any number of steps in these processes could lead to a breakdown in the Wnt/Wg-signaling circuit from signaling to target cells. These observations show that post-translational maturation of Wg, cell-surface components required for packaging and dispersal into the extracellular milieu, plays essential roles in the transmission of the Wg signal. In addition, plasma-membrane composition and organization, and the molecules that drive these parameters, are likely to be influential variables in the regulation of Wg cellular signaling.

The complexity of Wnt signal transmission is not limited to maturation of the ligand in the sending cells. It now appears obvious why Wg must interact with both its cognate receptor (Frizzled) and an LDL-like co-receptor (Arrow) for receipt of the Wg signal. Correct processing of Arrow in the receiving cells is also essential.
for Wg signal transduction (Culi and Mann, 2003). The processing of both Wg in the sending cells and its receptors in the receiving cells must occur for appropriate Wg presentation and a productive receptor interaction, which are required to activate the pathway. It has also been reported that Wg is differentially degraded in distinct types of receiving cells in embryos (Dubois et al., 2001; Piddini et al., 2005). Clearly, mechanisms governing this process could be controlled by events both in signaling and in target cells.

Here, we identify a new component in the Wg signaling pathway, sprinter (srt). Absence of srt results in the accumulation of the Wg ligand in signaling cells and in an inability to activate downstream targets of Wg signaling in receiving cells. Therefore, we hypothesize that srt encodes a factor required for Wg maturation as Wg moves through the secretory pathway to yield active ligand or a protein that promotes proper packaging and dissemination of Wg from the plasma membrane of signal-producing cells. Appropriate maturation of Wg in the Wg-producing cells could influence its compartment-targeting in the sending cells, targeting or binding specificity in receiving cells, or uptake and endocytic handling in receiving cells. Two recent papers also identify mutations in this locus, referring to the gene as wntless (wls) (Bänziger et al., 2006) or evenness interrupted (evi) (Bartscherer et al., 2006), and reach a similar conclusion regarding its role in Wg signaling.

MATERIALS AND METHODS

Fly strains

The following srt-mutant strains were used for germline clones and/or mosaic analysis: w; mwh srt7E4 FRT2A/TM3, Shw; srt7E4 FRT2A/TM6B, Twi and w; srt7E4 FRT2A/TM3, GAL4-twi2.3, UAS-2xEGFPAH2.3, Shw Ser.

Germline clones

srt germline clone embryos were generated by crossing y hs-flp/Y; P[oovoD1-18]SL FRT2A/TM3, Shw males to w; srt7E4 FRT2A/TM3 virgin females. Resulting third-instar larvae were then heat-shocked for 1 h at 37°C to induce Flp/FRT-mediated recombination, as previously described (Chou and Perrimon, 1996; Chou and Perrimon, 1992). The resulting y hs-flp/hc; P[oovoD1-18]SL FRT2A/srt7E4 FRT2A virgin females were then crossed to w; srt7E4 FRT2A/TM3 and Df(3L)vin5, ru hs hgl e ca/TM3, Shw Ser males to compare srt7E4 FRT2A/srt7E4 FRT2A germline embryo to srt7E4 FRT2A/Df(3L)vin4 (srt7E4) embryos. For cuticle preparation, 24- to 36-hour embryos were dissected in 50% bleach for 3 minutes, mounted in Hoyer’s media, heated to 55°C for 24 hours and visualized on a Zeiss Axiophot in darkfield. For antibody staining, embryos were dechorionated and fixed in 4% formaldehyde, as previously described (Patel, 1994).

Adult and larval clonal analysis

srt-mutant somatic clones were induced by heat-shock in first instar larvae (Golic, 1991; Xu and Rubin, 1993). Adult clones were generated by crossing w; mwh srt7E4 FRT2A/TM3, Shw males to y hs-flp/y hs-flp; M(3)35 hs-GFP FRT2A/TM3, Shw, and the wings from non-Sb adult progeny were mounted in Euparal (ASCO Laboratory) and inspected for clones with a Zeiss Axiohot in brightfield. The presence of mwh identified the homozygous srt7E4-mutant tissue. Marked larval clones in wing imaginal discs were obtained by crossing y hs-flp/y hs-flp; M(3)35 hs-GFP FRT2A/TM6B to w; srt7E4 FRT2A/TM6B, Twi (Baeq et al., 2004). Wing discs from Twi larvae were dissected 1 hour following a 1-hour heat-shock at 37°C to induce GFP expression. Dissected wing discs were then fixed in 4% formaldehyde and stained, and homozygous srt7E4-mutant tissue was visualized by the absence of GFP, as previously described (Baeq et al., 2004).

Antibody labeling

For embryo and wing-disc antibody staining, the following primary antisera were used: mouse anti-Wg (4D4; Developmental Studies Hybridoma Bank, DSHB) (Brook and Cohen, 1996) diluted 1:10; guinea pig anti-Sen (from Hugo Bellen, Baylor College of Medicine, Houston, TX) (Nolo et al., 2000) diluted 1:1000; mouse anti-Pc (SE10 from David Strutt, University of Sheffield, UK) diluted 1:3000; rat anti-Ci (from Robert Holmgren, Northwestern University, Evanston, IL) (Motzny and Holmgren, 1995), 1:10; mAb anti-Ac (DSHB) (Skeath and Carroll, 1991) diluted 1:3; rabbit anti-Hh (from Inge The, University of Massachusetts Medical School, Worcester, MA) (Taylor et al., 1993), diluted 1:200; and mouse anti-En (4D9, DSHB) (Patel et al., 1989) diluted 1:4. Fluorescent secondary antibodies from Jackson ImmunoResearch Laboratories and AlexaFlours from Molecular Probes were used at a dilution of 1:500. Discs were mounted in Vectashield mounting media (Molecular Probes) and inspected using a Zeiss LSM510 confocal microscope. Z-series projections were rendered as a maximum intensity projection using Zeiss LSM 510 software (version 3.2).

Sprinter-candidate identification

The srt mutation was uncovered by Df(3L)vin5 (68A2-3;69A1-3) but excluded from Df(3L)ED4457 (67E2;68A7) and Df(3L)vin4 (68B1-3;68F3-6), placing the mutation on the left arm of chromosome 3 within the cytological region 68A7 to 68B3. From these results, the left-most candidate open reading frame (ORF) in this region was identified as CG7628, a putative phosphate transporter, by the exclusion of srt from the molecularly defined deficiency ED4457. We were able to ascertain the right-most candidate ORF as CG6190 using quantitative PCR to define the left molecular endpoint for Df(3L)vin4 between CG6190 and CG7600, leaving 15 srt candidate ORFs within this region. As the germline clone screen that identified sprinter requires that the candidate transcript must be maternally loaded, we further eliminated candidates within this region by performing RT-PCR with RNA isolated from unfertilized embryos to determine which of the remaining srt candidates were maternally loaded. Among the remaining candidates, we found that six were not maternally loaded, leaving nine candidates. Utilizing P11739 localized to the 5’ UTR of Algo10/C32076 yielded mutations that were found to complement srt, eliminating this gene as a candidate and reducing the candidate pool to eight. Identification of srt as a nonsense mutation in CG6210, srt mRNA rescue of the mutant phenotype and srt RNAi induction of the mutant phenotype are described in the Results. Injection of GFP srt embryos with dsRNA targeted to three other ORFs among our remaining candidates (CG6207, CG6190 and CG7616) did not generate a segmentation defect (data not shown). Wild-type embryos with both maternal and zygotic srt gene expression injected with 543 bp srt dsRNA hatched with no apparent defects when compared to a dsRNA targeted to wg, a control zygotic target (data not shown). This suggests that the srt maternal contribution could not be knocked down sufficiently to yield in embryonic lethality.

Quantitative PCR

Homoygous-mutant GFP-negative Df(3L)vin4, ru h g e ca embryos were enriched by sorting 16- to 20-hour embryos from Df(3L)vin4, ru h g e ca/TM3, Shw, Twi-GFP under a fluorescence stereo microscope. Genomic DNA was prepared from both GFP (vin4) and GFP (vin3_TM3, Shw, Twi-GFP) embryos, and was used to perform Q-PCR in a BioRad iCycler using the QuantiTect SYBER Green PCR kit (Qiagen). DNA sequences present in the ORFs that are deleted from the vin4 chromosome are less represented using GFP than in GFP genomic DNA samples, resulting in a higher threshold of detection. Typically, an ORF was determined to be within vin4 if the ΔΔct between GFP and GFP genomic DNA samples was greater than three cycles. Primer sequences used in this experiment are available upon request. By these criteria, the left end point for the Df(3L)vin4 was defined by determining that CG6190 was outside of, and CG7600 was within, the deleted region of vin4.

RT-PCR

Total RNA was isolated from 16- to 20-hour unfertilized and control fertilized w1118 embryos using Ultraspec (Biotecx). Presence of the message from each ORF within 68A8-68C1 was detected with the Super Script One-Step RT-PCR kit (Invitrogen). Srt maternally loaded candidate ORFs were defined as those present in both unfertilized and fertilized embryos, as compared to zygotic controls that are only present in RNA derived from fertilized embryos. Evaluation of each ORF was determined from three independent RNA samples.
RNA rescue and RNA interference in srt^{7E4} germline clone embryos
Germine clone females generated in mass as described above were crossed to w/Y; srt^{7E4} FRT^{2A}/TM3, GAL4-uv5/TM{3}, UAS-2xEGFP^{1N2.1}, Sb Ser. Embryos from this cross 0- to 1-hour old were aligned on double-stick tape, desiccated, covered with halocarbon oil and injected at the midline with either srt mRNA or dsRNAs at a concentration of 1 mg/ml. Following an approximately 48-hour incubation period at 18°C, the embryos and any hatched larvae were scored for GFP fluorescence and separated into GFP+ and GFP- groups. The samples were briefly washed with heptane to remove the halocarbon oil, mounted in Hoyer’s media and visualized as above. srt polyadenylated message was prepared in vitro using mMessage mMachine T7 Ultra kit (Ambion) from linearized pOT2-GH01813 (full-length srt cDNA, isoform A). dsRNA against CG6210/srt, CG6207, CG6190, CG7616 and wg were prepared using the Megascript kit (Ambion) from PCR templates derived from w^{1118} genomic DNA. Primer sequences are available upon request.

RESULTS
sprinter is a mutation that alters epithelial patterning in embryos
The srt^{7E4} EMS mutation was initially identified in a ‘maternal effect’ screen that takes advantage of the dominant female-sterile FLP technique to generate germline clone embryos (Chou and Perrimon, 1996; Chou and Perrimon, 1992; Chou et al., 1993). Using this method, only those female germ cells in which both homologs of a gene are mutant are able to complete oogenesis and produce fully mature eggs. Hence, fertilization of these eggs by heterozygous mutant fathers allows us to monitor embryonic development in the absence of maternal and zygotic contribution of the gene in question. Examination of the cuticles from srt^{7E4} germline clone embryos, (hereafter referred to as srt^{7E4} embryos), results in the loss of naked cuticle to yield a ‘lawn of denticles’ phenotype (Fig. 1C), as compared with the normal patterning of wild-type embryos (Fig. 1A). As Wg and Hh signaling exist in a feedback loop during embryonic epidermal patterning, this result suggests that either the Wg or the Hh pathway is disrupted (Martinez-Arias et al., 1988; Perrimon, 1994). The srt^{7E4} phenotype can be rescued to larval and adult stages of development if the father contributes a wild-type copy of the gene, although some die during embryonic development with mild segment-polarity defects (Fig. 1B). srt^{7E4} is a strong loss-of-function mutation, because its embryonic cuticle phenotype is just as severe whether the father contributes a srt^{7E4} or srt^{7E4} chromosome (Fig. 1D). Zygotic srt^{7E4}/srt^{7E4} transheterozygotes are pupal lethal, suggesting maternally contributed Srt function perdures through the larval stages of development (see Fig. S2 in the supplementary material).

Loss of sprinter in embryos blocks signaling by preventing Wingless secretion
Because of the signaling feedback loop that exists between the Wg and Hh pathways during embryonic development, it is difficult to determine which pathway is specifically disrupted (DiNardo et al., 1988; Martinez-Arias et al., 1988; Heemskerk et al., 1991; Bejsovec and Martinez Arias, 1991). We examined the expression of Wg in both paternally and non-paternally rescued srt^{7E4} embryos, and found that Wg accumulated in the cells that express it (compare wild type Fig. 2A,D with Fig. 2B,C,E,F). This result indicates that mutation in srt obstructs normal Wg secretion and thus prevents normal Wg signaling to yield the embryonic ‘lawn of denticles’ phenotype (Fig. 1C,D).

Low levels of Wg secretion rescues downstream signaling in embryos
Because we had observed that paternally rescued embryos reached adult stages of development, we compared the Wg-secretion phenotypes at stages 9 and 13 of embryonic development in non-paternally and paternally rescued srt^{7E4} embryos with wild-type embryos (Fig. 2A,D). Non-paternal and paternal rescue was distinguished by the presence of the TM3, Twist-GFP balancer (data not shown). Surprisingly, we detected little difference in the accumulation of Wg in stage 9 non-paternally (Fig. 2B) and paternally (Fig. 2C) rescued srt^{7E4} embryos, although some Wg was detected in endocytic vesicles in the receiving cells in the paternally rescued srt^{7E4} embryos (Fig. 2C, arrowheads). Even by stage 13, when Wg had completely faded from epidermal cells and could only be detected in the neuroblasts of non-paternally rescued srt^{7E4} embryos (Fig. 2E) (Ingham and Hidalgo, 1993), Wg accumulation was still significant in paternally rescued stage-13 embryos (Fig. 2F). Through the later stages of embryonic development, paternally rescued srt^{7E4} embryos gradually approached, but never completely achieved, wild-type levels of Wg secretion (data not shown). Very little Wg is released from paternally rescued srt^{7E4} embryos, but this is sufficient to maintain expression of Engrailed (En), an epidermal target for Wg signaling in receiving cells (Fig. 3B) (Ingham and Martinez Arias, 1992; Perrimon, 1994), to ultimately yield larva capable of reaching adult stages of development. This suggests only a minimal level of

![Fig. 1. sprinter mutations yield a ‘lawn of denticles’ phenotype in germline clone embryos.](Image) Embryos orientated anterior left, posterior right and ventral or ventrolateral surface facing out. (A) A cuticle from wild-type embryos has a regular patern of denticles and naked cuticle. (B) A cuticle from srt^{7E4} germline clones that are paternally rescued show minor segmentation defects and often survive to adult stages of development. Non-paternally rescued srt^{7E4} germline clone embryos show a ‘lawn of denticles’ phenotype that is typical of loss of Wg- or Hh-signaling if the father contributes the srt^{7E4} chromosome (C) or srt^{4} (D) from Df(3L)win5. 

RESEARCH ARTICLE 4903
DEVELOPMENT
Fig. 2. Wg accumulates in the cells that express the ligand in srt7E4 embryos. Stage-9 (A-C) and stage-13 (D-F) embryos oriented anterior up, posterior down and ventral or ventrolateral surface facing out. (A,D) Expression of Wg in wild-type embryos shows a low level of Wg in Wg-expressing cells, and numerous Wg punctate endocytic vesicles in the receiving cells at both stages of development. (B) Wg accumulates in expressing cells in non-paternally rescued embryos at stage 9. Notice that punctate vesicles are absent and Wg fills expressing cells. (C) Similar retention of Wg is observed in srt7E4 paternally rescued embryos, although a few endocytic vesicles can be detected in receiving cells (arrowheads). (E) By stage 13, Wg has completely faded from the epidermal surface of non-paternally rescued srt7E4 embryos and can be detected only in neuroblasts. (F) In paternally rescued stage-13 srt7E4 embryos, there is still a significant level of Wg within the cells that express it, suggesting that the Wg-Hh-feedback signaling loop is still intact. Wg vesicles are also detected (arrowheads). The difference in background between wild-type and srt7E4 embryos is due to the higher laser intensity used to collect data from wild-type embryos, which is not required for the mutant. For a direct comparison of Wg levels between wild-type and srt7E4 mutant tissues see Fig. 6. All images are projections of three optical sections acquired at 1 μm z-intervals, except E, which is a single optical section. Scale bar: 20 μm.

secreted Wg is necessary to maintain downstream Wg signaling events required for embryonic patterning. En expression fades from the epidermis of stage 9 non-paternally rescued srt7E4 embryos (Fig. 3A) and is completely lost shortly thereafter (data not shown).

Loss of srt7E4 in the adult causes wing nicks
In order to address pathway specificity in a different context, we examined the effect of homozygous srt7E4-mutant tissue on the development of adult wings given that, during wing development, the Wg and Hh pathways operate independently. In the larval wing imaginal disc, Hh is expressed in the posterior compartment and signals anteriorly, promoting the appropriate patterning and growth of the intervein region between wing veins 3 and 4 in the adult (Méthot and Basler, 1999; Sánchez-Herrero et al., 1996; Slusarski et al., 1995). Wg is secreted from the dorsoventral boundary of the wing imaginal disc to mediate patterning of the wing margin (Couso et al., 1994). The effect of srt7E4 homozygous mutant tissue, marked by multiple wing hairs (mwh), was examined in adult wings (Fig. 4) (Golic, 1991; Xu and Rubin, 1993). We consistently observed that clones of srt7E4-mutant tissue at the wing margin resulted in the loss of both sensory bristles (Fig. 4B,D) and margin wing vein (Fig. 4C,E), a phenotype diagnostic of Wg-pathway disruption and consistent with our embryonic data. Large clones in the posterior wing, and those encompassing veins 3 and 4, which would be predicted to yield Hh-specific phenotypes, appeared normal, suggesting that the loss of srt7E4 does not disrupt Hh signaling in this tissue (data not shown). Furthermore, except for the wing-margin defects, we observed no other effect of srt7E4-mutant tissue on wing development. These results suggest that srt blocks Wg signaling during wing development.

The srt7E4 mutation blocks Wg signaling by preventing Wg secretion in wing discs
We further explored the effect of the srt7E4 mutation on Wg signaling by examining the expression of molecular markers in third instar larval wing discs. In this tissue, Wg is secreted from the dorsoventral boundary (Fig. 5A), and diffuses dorsally and ventrally to activate Senseless (Sen; Fig. 5B, blue) (Nolo et al., 2000) and Achaete (Ac; Fig. 5J, red) (Couso et al., 1994; Phillips and Whittle, 1993) in adjacent target cells. We generated srt7E4-mutant tissue in third instar wing discs, which were distinguished by the absence of GFP (Fig. 5F,I,M,N, green) (Golic, 1991; Xu and Rubin, 1993). Fig. 5D,G shows the side-by-side expression pattern of Wg in wild-type (within outline) and srt7E4-mutant (excluded from outline) tissue at the dorsoventral boundary. In srt7E4-mutant tissues, the intensity of Wg staining is significantly higher because the protein is accumulating in the cells that express it, as was observed in embryos. There is also a loss of Sen expression in the adjacent receiving cells (Fig. 5E,H), as compared with wild-type tissue (Fig. 5B). However, some srt7E4-homozygous Sen-expressing mutant cells are detected within the clone, suggesting that srt is not required in target cells to receive the Wg signal (Fig. 5E, excluded
from outlined heterozygous cells and Fig. 5H, arrows). These cells do not express Wg (Fig. 5G, arrows), confirming that they are exclusively target cells. In the anterior compartment, we do detect some low-level Sen expression in target cells that abut srt\textsuperscript{TE4}-mutant Wg-signaling cells, indicating that srt\textsuperscript{TE4} may not completely block Wg signaling (Fig. 5E). Similar results were obtained for Ac, a Wg anterior compartment target (Couso et al., 1994; Phillips and Whittle, 1993). Ac expression is lost when srt\textsuperscript{TE4}-mutant tissue crosses the Wg-expressing cells at the dorsoventral boundary (Fig. 5K, red). These results demonstrate that the action of Srt is non-cell autonomous, which would be expected for a gene product acting on a secreted ligand. Hence, the extracellular movement of Wg produced from a few srt\textsuperscript{TE4}-mutant Wg-producing cells, thereby preventing the activation of Wg downstream targets in adjacent receiving cells.

A projection of optical sections through a wing disc shows significant accumulation of Wg in the cells expressing the ligand at the dorsoventral boundary (Fig. 5M-O), as observed in embryos (Fig. 2). It is also clear that there are no detectable endocytic vesicles in adjacent target cells, which are present next to heterozygous Wg-secreting cells (Fig. 5M). In addition to a high level of Wg protein in srt-mutant cells, examination of the projection shows an expansion in the number of cells at the dorsoventral boundary that express Wg. This expansion in Wg expression occurs at the transcriptional level, as similar results were observed in srt\textsuperscript{TE4}-mutant clones for \textit{wg-lacZ} (see Fig. S1 in the supplementary material). This is probably due to an inability of adjacent dorsal and ventral cells to downregulate Wg expression in the process of self-refinement (Rulifson et al., 1996) (Fig. 5M-O). Notably, the large clones of srt\textsuperscript{TE4} encompassing almost an entire compartment did not distort the overall wing-disc structure, suggesting that srt is not essential for cell survival or cell proliferation in the wing pouch (Fig. 5F-O). If srt were necessary in the wing disc for Hh or other signaling pathways, such as Decapentaplegic (Dpp), such large clones would be expected to have a significant effect on cell growth and, as a result, on wing-disc morphology.

\textbf{srt\textsuperscript{TE4} has no effect on Hh secretion or on the activation of the downstream targets of Hh in wing discs}

In order to confirm that srt is specific for Wg secretion and signaling, we examined Hh expression and the activation of its downstream targets in the wing disc (Fig. 6). The anterior compartment and anterior-posterior compartment boundary is defined by the expression of Cubitus interruptus (Ci; Fig. 6B,E) (Han et al., 2005). In contrast to Wg, Hh does not accumulate in posterior srt\textsuperscript{TE4}-mutant ligand-expressing cells. Furthermore, we observe no disruption in Hh targeting to the anterior receiving cells, visualized as punctate staining in the Ci-expressing cells, regardless of whether the Hh was derived from or received by wild-type or srt\textsuperscript{TE4}-mutant cells (Fig. 6A,D). This result indicates that srt is not required for the delivery or the receipt of the ligand in efficient Hh signal transduction. This conclusion is supported by the fact that the anterior expression of Patched, a target of Hh signaling in the wing disc, is similar to wild-type expression (Fig. 6G) (Capdevila et al., 1994; Strigini and Cohen, 1997) whether posterior Hh-expressing cells or anterior Hh-receiving cells are wild type or srt\textsuperscript{TE4} mutant (Fig. 6H). Similar results were obtained for the anterior compartment expression of Engrailed, another target of Hh signaling in the wing disc (data not shown) (Blair, 1992; Blair and Ralston, 1997). Based on these results, we conclude that srt does not play a role in any aspect of the Hh signal transduction pathway in the wing disc. Therefore, we propose that the embryonic ‘segment polarity’ defects observed in the srt mutant result from Wg-signaling defects.

\textbf{srt\textsuperscript{TE4} is a nonsense mutation in CG6210}

Deficiency mapping placed the srt\textsuperscript{TE4} mutation on the left arm of chromosome 3 within the cytological region 68A4 to 68B3. Based on mapping and maternal-expression data (see Materials and methods), we reduced our candidates within this region to eight. Sequence analysis of genomic DNA from srt\textsuperscript{TE4}/TM3 heterozygous adults showed that the CG6210 open-reading frame (which is expressed in a dynamic pattern shown at http://www.fruitfly.org/cgi-bin/ex/basic.pl?find=CG6210&submit=Run%20Basic%20Query) from the srt\textsuperscript{TE4} chromosome had a G to A transition yielding a
nonsense mutation at nucleotide +1572 of the srt cDNA, converting a tryptophan (TGG) to a stop (TGA) (Fig. 7). This mutation is predicted to remove the last 71 amino acids from the protein encoded by CG6210/srt. In order to confirm that CG6210/srt was in fact the gene disrupted to yield the embryonic ‘lawn of denticles’ phenotype, we generated srt7E4 germine clone females and crossed them to srt7E4/TM3,Sb,Twi-GFP. Zygotic expression of GFP from this balancer allows unambiguous identification of embryos receiving the srt7E4 chromosome (GFP–) from those receiving the srt wild-type chromosome (GFP+) from their father. The embryos derived from this cross were used in two microinjection experiments. First, these embryos were injected with CG6210 mRNA prepared in vitro from the srt cDNA (GH01813) to rescue the ‘lawn of denticles’ phenotype of GFP embryos. These

Fig. 5. srt7E4 mitotic clones result in Wg accumulation at the dorsoventral boundary and blocks the activation of targets in Wg receiving cells in the wing disc. The wild-type expression pattern for Wg (red; A) and its downstream targets Senseless (Sen, blue; B) and Achaete (Ac, red; J) in third instar wing imaginal discs. (C) Images A and B merged with wild-type Green Fluorescent Protein (GFP). (D-I) The effects of srt7E4 homozygous mutant tissues on the expression of Wg and Sen at two different magnifications. (K-L) Anterior Ac expression. srt7E4-homozygous-mutant cells are marked by the absence of GFP shown in merged images (F,J,L,N). Retention of Wg within Wg-expressing cells at the dorsoventral boundary blocks dorsal and ventral expression of Sen (E,H). Sen is expressed when GFP-positive (heterozygous) cells are present at the dorsoventral boundary, but does not require wild-type srt because many srt7E4-homozygous cells are present (Sen-expressing cells excluded from outlined region in E and H). Arrows in G and H show two srt7E4-mutant cells that express high levels of Sen, but not Wg. Similarly, anterior Achaete expression also requires a wild-type srt in Wg-expressing cells at the dorsoventral boundary (K,L). (M-O) A projection of optical sections of a Wg-stained (red) third instar wing disc shows the dramatic difference in the presence of Wg within the cells that express it between srt7E4-mutant (left side) and heterozygous tissue (right side). Homozygous mutant cells are identified by the absence of GFP (O). Punctate Wg endocytic vesicles (arrowheads) are present adjacent to Wg-expressing heterozygous cells and absent near srt7E4-homozygous tissue. There also appears to be an expansion in the number of cells that express Wg at the dorsoventral boundary when srt function is absent. All wing discs are orientated dorsal up and anterior left. Images A-I are single optical sections focusing on the Sen-expressing nuclei. (J-O) Projection of eight optical sections acquired at 1 μm z-intervals. Scale bars: 20 μm.
injections resulted in partial or complete rescue of the ‘lawn of denticles’ phenotype, as compared with buffer-injected controls, suggesting that the presence of the srt message specifically rescued embryos lacking maternal and zygotic srt (Fig. 7A). Next, these embryos were injected with a 543 bp double-stranded RNA (dsRNA) targeted to the srt message. In this experiment, we looked for recapitulation of the ‘lawn of denticles’ phenotype in the GFP+ embryos. We found that srt RNA interference in GFP+ embryos, which are often rescued to survive to larval stages of development or beyond, now died during embryonic development and had a ‘lawn of denticles’ phenotype analogous to that of srt7E4 embryos (Fig. 7B).

Imprecise excisions of two viable P-elements mapping to the 5′ untranslated region (UTR) of CG6210/srt – P[EPgy2]EY01593 and P[EPgy2]EY11001 – has yielded six srt7E4 non-complementing lethal excisions that are large chromosomal deletions to define six new srt alleles. srt7E4 is a deletion that removes the entire 5′ UTR of CG6210/srt, as well as most of exon 1 (up to nucleotide +901), and hence is probably a null allele of srt. Phenotypic characterization of the srt-deletion mutants show that they display phenotypes identical to that observed for srt7E4 (see Fig. S2 in the supplementary material).

**Sprinter is predicted to encode an evolutionarily conserved multi-transmembrane-spanning protein of unknown function**

The srt/CG6210 genomic locus is composed of three exons with two possible splice variants to encode novel proteins of 594 (isofrom A) and 562 (isofrom B) amino acids that include or exclude exon 2. Both splice variants are expressed in Drosophila, as the EK288129 and CK00022 ESTs exclude the second intron, whereas the srt GH01813 cDNA used in our rescue experiment includes it. Indeed, we have found that both splice variants are expressed in S2R+ cells (data not shown). Our analysis of the amino acid sequence suggests that Srt is composed of four to eight transmembrane domains. The signal sequence constitutes the first transmembrane domain because it does not have a good consensus-signal peptidase-cleavage site (Bendtsen et al., 2004). The next four hydrophobic sequence elements all represent potential transmembrane domains, but are either too short to traverse the membrane or are weakly hydrophobic, reducing the likelihood that they are within the membrane (Fig. 7E, gray bars). The next three hydrophobic regions of the Srt protein are probably transmembrane domains (Fig. 7E, black bars). Based on these observations, we hypothesize that Srt has four transmembrane domains with a large N-terminal globular extracellular/luminal domain that has two potential N-linked glycosylation sites (Fig. 7D), although several other topologies are clearly possible (Puntervoll et al., 2003). This predicted structure places the Trp492 of the Srt protein sequence within the last transmembrane domain to yield either a truncated protein or one that is earmarked for degradation through nonsense-mediated decay of the message or through the breakdown of the misfolded protein. We also noticed that, although Flybase (www.flybase.org) has srt/CG6210 annotated as a multi-drug-resistance-related protein (MRP), our analysis of the Srt amino acid sequence indicates that the only commonality between these proteins is that they are multi-transmembrane-spanning proteins. Hence, the current annotation of srt/CG6210 in Flybase as MRP is incorrect.

Sequence comparison of Srt to all protein databases reveals that its closest known relative is found in Drosophila pseudoobscura sharing 87% identity and 91% similarity along its length. In Drosophila melanogaster, the closest relative of Srt is encoded by CG13409, located at cytological region 94A, and has only 22% identity and 42% similarity. Srt shows much stronger homology to protein sequences from its evolutionarily distant relatives, suggesting that Srt is unique in Drosophila. Fig. 8E shows the alignment of the Drosophila Srt isoform B relative to nematode, frog and human. Overall, the Drosophila Srt isoform B shares 43% identity and 62% similarity with human Srt (hSrt). Whereas some regions in the N-terminus and the majority of C-terminal regions of Srt diverge from its vertebrate relatives, there is a high level of conservation that extends throughout the central
region of Srt. The most N-terminal amino acids, including the signal sequence/first putative transmembrane domain, are fairly well conserved across species, even in the absence of a good consensus peptidase cleavage site, supporting the hypothesis that this constitutes a transmembrane domain (Bendtsen et al., 2004).

**DISCUSSION**

This paper describes the characterization and cloning of *sprinter*, a novel transmembrane protein required for Wg signal transduction. Our results demonstrate that the absence of wild-type *srt* in germline clone embryos and wing discs results in the accumulation of Wg protein within the Wg-producing cells. As the signaling cells are not

---

Fig. 7. *srt* encodes an evolutionarily conserved multi-transmembrane protein. (A) Injection of *srt* mRNA into non-paternally rescued germine clone embryos rescues the segmentation defect (right). The buffer-injected control shows the null *srt* phenotype. (B) Injection of a 543 bp *srt* dsRNA into GFP+ paternally rescued *srt* embryos yields a cuticle phenotype (right) almost as severe as the buffer-injected non-paternal rescued embryo in A and Fig. 1C. (C) The *srt* genomic region located at 68A9 is a gene-dense region. The *srt* ORF is flanked closely by Alg10 (left) and shares its 5' proximal region with CG7616 (right; www.flybase.org). The *srt* ORF is composed of two or three exons to yield two splice variants. (D) One possible topological model for the protein encoded by *srt*: the luminal/extracellular region, above; the cytoplasmic region, below. Four putative transmembrane stretches are shown. The 7E4 mutation in *srt* results in a nonsense mutation in the fourth transmembrane span in our model. The location of the splice variant in the intracellular loop and possible sites of N-linked glycosylation are shown. (E) Sequence alignment of *Drosophila srt* B isoform (NCBI accession: NP_729681) with its homologs from *C. elegans* (NP_001022275), *X. laevis* (AAH81130) and human (NP_079187). The four transmembrane domains shown in D are underlined in black. Four possible additional transmembrane domains are underlined in gray. The asterisk under the last transmembrane marks the location of the *srt*7E4 mutation.
able to release functional Wg protein, activation of downstream Wg targets, such as maintaining En expression in embryos or activating the expression of Sen and Ac in wing imaginal discs, does not occur normally in the receiving cells. We also show that srt-mutant Wg target cells can express downstream targets of the pathway – Sen and Ac – which demonstrates that srt is not required in the target cells to receive or transduce the Wg signal. srt is apparently specific for the Wg pathway, as we detected no defect in Hh signaling regardless of whether the Hh ligand originated from or is received by srt724-mutant tissues. Furthermore, we observed no adult phenotypes that indicated that other signaling pathways are disrupted.

**Srt function**

Based on our results, we believe that the primary function of Srt in the Wg pathway is to support the maturation of activate Wg ligand. In this capacity, it is possible that Srt acts in post-translationally processing Wg, in the targeting of Wg to the plasma membrane or in the release of active Wg from the membrane. As porcupine mutants, as well as point mutations in the Wg protein itself, yield similar Wg-retention phenotypes (Manoukian et al., 1995; Dierick and Bejsovec, 1998; van den Heuvel et al., 1993), and because porc is required for the post-translational processing of Wg (Kadowaki et al., 1996; Tanaka et al., 2002; Zhai et al., 2004), a role for Srt in the post-translational processing of Wg is one possible function of Srt.

In this role, we would predict that Srt might act as an enzyme that either participates in known post-translational changes to the Wg protein, such as glycosylation or palmitoylation, or identifies a new post-translational alteration in Wg that is required for its maturation. In addition to catalyzing the palmitoylation of Wg, the action of Porc is required to target Wg to lipid rafts in the plasma membrane (Zhai et al., 2004). This observation suggests that membrane targeting might occur by an active process mediated by specific protein(s). Another possible function of Srt could be as a Wg-specific chaperone protein that promotes proper folding and shuttles Wg through the secretory pathway to the plasma membrane once post-translational processing is complete. Indeed, there is precedent for the need of protein-specific chaperones in the Wg pathway. In order for functional Arrow – the Wg low-density lipoprotein co-receptor – to reach the plasma membrane, it requires the activity of a specific chaperone protein, Boc (Culi and Mann, 2003). Recent studies suggest that at least some Wg protein is loaded into lipoprotein particles during larval development (Panáková et al., 2005), which may be required for the movement of lipid-modified Wg in the extracellular space to establish its morphogenetic gradient in the wing. These lipoprotein particles are exogenously synthesized in the fat body and must be loaded with their lipid-modified cargo in the cells that express the ligand (Panáková et al., 2005). Hence, there must be protein(s) present at the plasma membrane that catalyzes this process. Sprinter may be localized within membrane rafts, at the ready to load palmitoylated Wg into arriving lipoprotein particles for dissemination to the Wg target cells. Another potential role for Sprinter could be to act indirectly on Wg by supporting the post-translational maturation or subcellular targeting of the proteins that directly regulate these processes, although a physical interaction between Wg and Srt (also known as Wls or Evi) has been reported (Bänziger et al., 2006). As a Wg-specific secretory chaperone, numerous other functions of Srt could be imagined, and we are actively trying to determine the specific role of Srt among these possibilities.

Localization of Srt within the secretory pathway could be predictive of its function. Srt localization in lipid rafts at the plasma membrane would suggest involvement in generating Wg-loaded lipoprotein particles. However, ER or Golgi localization could indicate a role in Wg maturation as it moves through the secretory pathway. Although determination of the subcellular localization of Srt awaits specific antibodies, we have observed that, in srt724-mutant tissues, there is shift in the cellular distribution of Wg toward the basolateral surface of wing-disc cells – the surface of Wg extracellular gradient formation in target cells (Baeg et al., 2004; Greco et al., 2001; Strigini and Cohen, 2000) without disruption of Golgi localization. This would suggest that the srt block to Wg maturation occurs within the ER or a post-Golgi compartment of the secretory pathway (E.M.S., unpublished).

**srt specificity to the Wg pathway**

We have found that the activity of the protein encoded by srt disrupts the Wg pathway, but has no effect on Hh signaling. During wing-disc development, Hh is expressed in the posterior compartment and moves anteriorly to activate its anterior-compartment targets that promote cell growth and patterning of the wing (Méthot and Basler, 1999; Sánchez-Herrero et al., 1996; Slusarski et al., 1995). In our analysis of the developing wing, there is no indication that srt plays a role in Hh signaling. Large clones encompassing the adult wing blade show no alteration in the separation of wing veins 3 and 4 observed in Hh-signaling mutants. Furthermore, homozygous mutant wing discs from a probable srt-null allele show normal wing-disc size and morphology. In the wing disc, there was no detectable accumulation of Hh in the srt724-mutant cells that express it, as was observed in Wg-producing cells. Additionally, we found that Hh is visualized in its anterior-compartment receiving cells within punctate vesicles typical of Hh in wild-type tissues (Tabata and Kornberg, 1994). Regardless of whether the protein was derived from or received by srt724-mutant cells, the distribution of Hh in the wing disc appeared similar to wild-type cells. Furthermore, we also looked at the anterior-compartment expression of Ptc, a sensitive readout for Hh target cells in the wing (Capdevila et al., 1994; Strigini and Cohen, 1997). Again, no matter where the srt724 homozygous mutant tissue was located with respect the anterior-posterior axis, Ptc expression looked wild type. Therefore, at both the level of ligand expression and activation of downstream targets, we found no indication that srt functions in Hh signal transduction during wing-disc development. Although our data do not directly address the possible role of srt in Hh signaling during embryonic development, based on these observations in wing discs it appears unlikely that srt affects Hh signaling during embryonic development. Hence, we hypothesize that the phenotypes observed in the developing embryo are specifically due to disruption of the Wg pathway.

Establishing the specificity of srt for Wg is important because lipid-modified ligands, such as Wg and Hh, have been shown to be present in the form of lipoprotein particles (Panáková et al., 2005). As one possible function of Srt could be the targeting or loading of Wg into lipoprotein particles for dissemination to receiving cells, Srt might serve a similar function for the dissemination of lipid-modified Hh to receiving cells. The fact that our results support the conclusion that srt is specific to Wg maturation would argue that this is not the case. These observations suggest that either Srt does not play a role in this process or that Wg and Hh use different protein assemblies to support this function. Analogous to the relationship between srt and Wg, Hh accumulates in dispatched (disp) mutant Hh-expressing cells (Burke et al., 1999). Perhaps Srt and Disp serve similar roles in the maturation of active Wg and Hh ligand, respectively. Lack of an effect on Hh secretion shows that Srt is not a general secretion factor for this class of ligands. It was also
important to demonstrate specificity for the Wg pathway, as srt^{2467}
was isolated in an embryonic screen (Chou and Perrimon, 1996) and
selected based upon its ‘lawn of denticle’ phenotype, which could
either arise as a result of the loss of Wg or Hh signaling.

Although we have demonstrated that srt is not required for Hh
signaling, it is possible that srt might be required in other signal
transduction pathways. Examination of adult clones revealed no
indication that srt disrupts other signaling pathways. However, it is
likely that srt is required for the maturation of other Drosophila
Wnts – a conclusion supported by the finding that multiple human
Wnts depend on Srt function (Bartscherer et al., 2006; Bänziger et
al., 2006).

We are grateful to Norbert Perrimon for performing the germline clone
that identified spiret. Special thanks to Kirk Czymmek for help with confocal
microscopy; Mary C. Farach-Carson and Reid Gilmore for assistance with
amino acid sequence analysis; and Vladic Mogila for preliminary mapping data.
We also thank H. Bellen, G. Boekhoff-Falk, I. Duncan, R. Holmgren, D. Strutt, I.
The, The Bloomington Stock Center and The Iowa Developmental Studies
Hybridoma Bank for reagents; and C. Cooper, R. Duncan, X. Lin, B. Storanch
and M. Feuiller for comments on the manuscript. This work was supported by a
March of Dimes Basil O'Connor Starter Scholar Research Award; a University
Hybridoma Bank for reagents; and C. Cooper, R. Duncan, X. Lin, B. Stronach
for amino acid sequence analysis; and Vladic Mogila for preliminary mapping data.

Although we have demonstrated that srt is not required for Hh
signaling, it is possible that srt might be required in other signal
transduction pathways. Examination of adult clones revealed no
indication that srt disrupts other signaling pathways. However, it is
likely that srt is required for the maturation of other Drosophila
Wnts – a conclusion supported by the finding that multiple human
Wnts depend on Srt function (Bartscherer et al., 2006; Bänziger et
al., 2006).

Development 133 (24)

References

(2006). Wingless morphogen gradient is established by the cooperative
action of Frizzled and Heparan Sulfate Proteoglycan receptors. Dev.
Biol. 290, 89-100.

Bänziger, C., Soldini, D., Schütz, C., Zippen, P., Hausmann, G. and Basler,
K. (2006). Wingless and Dally-like shape the extracellular Wingless

Bejsovec, J., DiNardo, S., Sher, E., Heemskerk-Jongens, J., Kassis, J. A.
and Ingham, P. W. (1993). Regulation of wingless transcription in the

development by regulating the activities of distinct transcriptional activator and

reticulum protein required for wingless signaling and trafficking of LDL
receptor family members in Drosophila. J. Biol. Chem. 278, 509-517.


Bott, L. and Brinkmann, A. (1992). Engrailed expression in the anterior lineage

reticulum protein required for wingless signaling and trafficking of LDL
receptor family members in Drosophila. J. Biol. Chem. 278, 509-517.

Bott, L. and Brinkmann, A. (1992). Engrailed expression in the anterior lineage

reticulum protein required for wingless signaling and trafficking of LDL
receptor family members in Drosophila. J. Biol. Chem. 278, 509-517.

reticulum protein required for wingless signaling and trafficking of LDL
receptor family members in Drosophila. J. Biol. Chem. 278, 509-517.
Role of sprinter in Wingless signaling


