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Directionality of Wingless protein transport influences epidermal patterning in the *Drosophila* embryo

Melissa M. Moline, Cozumel Southern and Amy Bejsovec*

Department of Biochemistry, Molecular Biology and Cell Biology, Northwestern University, 2153 Sheridan Road, Evanston, IL 60208-3500, USA
*Author for correspondence (e-mail: bejsovec@nwu.edu)

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SUMMARY

Active endocytotic processes are required for the normal distribution of Wingless (Wg) protein across the epidermal cells of each embryonic segment. To assess the functional consequences of this broad Wg distribution, we have devised a means of perturbing endocytosis in spatially restricted domains within the embryo. We have constructed a transgene expressing a dominant negative form of *shibire (shi)*, the fly dynamin homologue. When this transgene is expressed using the *GAL4-UAS* system, we find that Wg protein distribution within the domain of transgene expression is limited and that Wg-dependent epidermal patterning events surrounding the domain of expression are disrupted in a directional fashion. Our results indicate that Wg transport in an anterior direction generates the normal expanse of naked cuticle within the segment and that movement of Wg in a posterior direction specifies diverse denticle cell fates in the anterior portion of the adjacent segment. Furthermore, we have discovered that interfering with posterior movement of Wg rescues the excessive naked cuticle specification observed in *naked (nkd)* mutant embryos. We propose that the *nkd* segment polarity phenotype results from unregulated posterior transport of Wg protein and therefore that wild-type Nkd function may contribute to the control of Wg movement within the epidermal cells of the segment.

Key words: Wingless, Dynamin, *shibire*, Transcytosis, Pattern formation, *Drosophila*

INTRODUCTION

Wingless (Wg) belongs to the Wnt family of secreted growth factors (reviewed in Nusse and Varmus, 1992; Dierick and Bejsovec, 1999) and is the ortholog of vertebrate Wnt-1 (Rijsewijk et al., 1987). Wnts have been identified in a wide range of vertebrate and invertebrate species and have been implicated in numerous cell fate decisions during the development of these organisms. In *Drosophila*, Wg influences two distinct cellular decisions in patterning the larval ventral epidermis. This segmentally repeating pattern consists of six rows of uniquely shaped denticles arranged in a belt at the anterior of the segment and an expanse of smooth, naked cuticle in the posterior portion of the segment (Campos-Ortega and Hartenstein, 1985). In the absence of *wg* both the generation of diverse denticle types and the specification of naked cuticle are disrupted, resulting in a lawn of uniform denticles (Nüsslein-Volhard et al., 1984; Baker, 1988a). *wg* is expressed in one row of cells in each wild-type segment, roughly in the middle of the naked cuticle region (Baker, 1988b; Dougan and DiNardo, 1992). Thus Wg activity influences cell fate decisions many rows of cells away from its source.

Proper pattern formation requires temporal as well as spatial control of Wg activity (Bejsovec and Martinez-Arias, 1991; Heemskerk et al., 1991). Analysis of a temperature-sensitive *wg* allele that is wild type at 18°C and null for function at 25°C has shown that Wg activity between 4 and 5.5 hours of development generates diverse denticle types and stabilizes the expression of *engrailed*(*en*). *en* is a segment polarity gene expressed in the two rows of cells just posterior to the *wg* domain, at the posterior boundary of each segment. After 6 hours, Wg activity no longer produces these cellular responses, but instead promotes the naked cuticle-secreting cell fate. Thus the population of cells responding to Wg activity changes during development.

Wg and Wnt molecules tightly associate with membrane and extracellular matrix (Bradley and Brown, 1990; Papkoff and Schryver, 1990) and appear not to be readily soluble (Nusse et al., 1997). Thus, it is unlikely that these proteins freely diffuse through extracellular spaces. Rather, Wg appears to be transported via active cellular processes. This phenomenon was first demonstrated using the *shibire^{ts} (shi^{ts})* mutation to block endocytosis (Bejsovec and Wieschaus, 1995). *shi* encodes the fly dynamin homologue, a GTPase required for clathrin-coated vesicle formation (van der Bliek and Meyerowitz, 1991; van der Bliek et al., 1993). Rather than the broad, punctate Wg protein distribution normally found over several cell diameters on either side of the *wg*-expressing cells (van den Heuvel et al., 1989; Gonzalez et al., 1991), *shi* mutant embryos show high level accumulation of Wg around the *wg*-expressing cells.

This restricted distribution of Wg protein correlates with a

restriction in Wg signaling activity, as measured by stabilization of Armadillo (Arm) protein (Riggleman et al., 1990; Peifer et al., 1991). Activation of the Wg signal transduction cascade results in stable accumulation of Arm, which then forms a bipartite transcription factor with the HMG-box protein *Drosophila* TCF (dTCF) to drive expression of Wg responsive genes, such as *en* (Brunner et al., 1997; Riese et al., 1997; van de Wetering et al., 1997). *shi* mutant embryos show stripes of Arm staining that are similar to wild-type in intensity but are much narrower in width (Bejsovec and Wieschaus, 1995). *en* expression in the cells adjacent to the *wg*-expressing cells in these mutants is normal, indicating that *shi* disrupts neither Wg secretion nor signal transduction, and confirming that the Wg that accumulates in the extracellular space around the *wg*-expressing cells is functional. Thus, endocytosis is required for the wild-type distribution of Wg protein and for its consequent broad range of activity across the segment.

Structure/function analysis of the Wg molecule further supports the idea that active transport of the ligand is essential. Four mutations within *wg* have been isolated that specifically disrupt Wg transport without abolishing signaling activity (Hays et al., 1997; Dierick and Bejsovec, 1998). These mutant molecules generate a restricted response within the segment, as assayed by both cuticular pattern elements and molecular events. Homozygous mutant embryos produce naked cuticle but little denticle diversity, and show narrowed domains of Wg protein distribution and Arm stabilization. Three of these four mutations are single amino acid substitutions; each affects a residue that is highly conserved throughout the Wnt family (Dierick and Bejsovec, 1998), suggesting that ligand transport may be an important general aspect of Wnt function.

Here, we demonstrate that directionality of Wg movement is critical for generating the wild-type epidermal pattern. We have taken advantage of a dominant negative form of *shi* to perturb endocytosis in defined regions of the segmented embryonic epidermis. This technique allows us to assess cuticular pattern elements, which was not possible with the original shi^{ts} allele. Even brief exposures to the restrictive temperature during embryogenesis resulted in cell lethality and a failure to secrete cuticle (Bejsovec and Wieschaus, 1995). Similarly, high level expression of the dominant negative *shi* transgene in embryos leads to cell death and defects in cuticle deposition. However, moderate level expression does not affect cell viability and instead appears to produce fairly specific disruption of Wg ligand transport through the affected domain of cells. Embryos expressing such constructs survive to secrete cuticle, allowing us to link the molecular consequences of restricting Wg protein distribution with its ultimate effect on specification of epidermal cell fates.

MATERIALS AND METHODS

Drosophila stocks and culture conditions

Fly stocks were cultured at 25°C on standard cornmeal/yeast medium or on apple juice agar plates for egg collection. wg^{CX4} is a null allele that produces no detectable *wg* RNA (Baker, 1987). nkd^{7E89} is a strong allele of *nkd*, presumed to be null or close to null in function (Bejsovec and Wieschaus, 1993). The *en-Gal4* and *prd-Gal4* driver lines are described in Yoffe et al. (1995); the *E22C-Gal4*, *wg-Gal4* and *UAS-*wg^{+} are described in Hays et al. (1997); the *UAS-dTCF-ΔN* is described in Cavallo et al. (1998) and the *UAS-Dfz2* is described in Cadigan et al. (1998).

Transgene construction and analysis

The dominant negative mutant form of dynamin, containing a lysine-to-alanine substitution in the GTP-binding domain, fails to bind or hydrolyze GTP and blocks receptor-mediated endocytosis when overexpressed in tissue culture cells (van der Bliek et al., 1993). The *K44A* mutation was engineered into *shibire* by Alex van der Bliek, who kindly sent us the clone. We subcloned this shi^{D} sequence into a pUAST vector (Brand and Perrimon, 1993), which allows transgene expression to be directed by *Gal4* driver lines. Plasmid DNA was purified by Qiagen column preparation and microinjected into stage 2 embryos. Ten independent germline transformants were recovered and tested for transgene expression level. Level of expression was determined empirically by comparing phenotypic effects with those of the original shi^{ts} mutation. Lines producing high levels of transgene expression show cell lethality and defects in cuticle deposition. Lines producing moderate expression levels do not show cell lethality, unless driven with the strong ubiquitous *E22C-Gal4* driver, and so presumably do not block endocytosis completely. Cuticle deposition appears normal in such embryos, allowing analysis of cuticular pattern elements. Experiments were performed with lines expressing the transgene at moderate levels, unless otherwise noted.

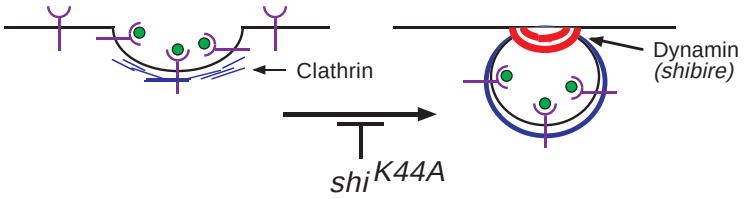
Antibody staining and cuticle preparation

For cuticle preparation, embryos were allowed to age at 25°C, dechorionated and mounted in Hoyer's medium/lactic acid (Wieschaus and Nüsslein-Volhard, 1986). Antibody staining was as described in Dierick and Bejsovec (1998). Anti-Wg antibody was used at 1:1000 (gift from S. Cumberledge), anti-En was used at 1:50 (gift from M. Peifer), anti-Neurotactin was used at 1:4 (gift from Y. Hiromi) and anti-Arm was used at 1:400 (gift from E. Wieschaus). Wingless/Neurotactin double-labeling was performed with mixed primary antibodies and mixed secondary antibodies (Boehringer Mannheim). Embryos were mounted in Aquapolymount (Polysciences) and viewed with a Biorad MRC600 confocal microscope.

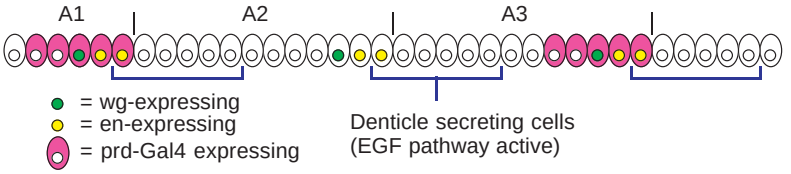
RESULTS

Perturbing endocytosis in defined domains restricts Wg protein transport and signaling activity

We have constructed a *Gal4*-driven *UAS* transgene that expresses a dominant negative form of *shi*, the fly dynamin. Dynamin is essential for receptor-mediated endocytosis (Fig. 1A; van der Bliek et al., 1993). Overexpression of mutant dynamin interferes with function of the endogenous wild-type molecule and leads to arrest of clathrin-coated pit constriction and detachment (Damke et al., 1994). Thus we can disrupt the normal endocytotic process by driving expression of the shi^{K44A}*(*shi^{D}*)* construct ectopically. High level or ubiquitous expression of this transgene phenocopies the effects of the original shi^{ts} mutation, abolishing cuticle deposition. However, moderate level, spatially controlled expression of the transgene does not perturb cuticle deposition and permits analysis of cuticular patterning. We use three *Gal4* driver lines to express the transgene in distinct subdomains of the epidermal epithelium (Fig. 1B): *wg-Gal4* and *en-Gal4*, which drive expression in each segment, and *prd-Gal4*, which drives expression in odd-numbered abdominal segments in a 5-cell-wide domain containing the *wg*- and *en*-expressing rows of



A. Receptor-mediated endocytosis is disrupted by dominant negative Shibire



B. *prd-Gal4* drives *shi*^{D} expression in defined domains within the epidermis

Fig. 1. Schematic diagram of *shibire* function and domains of transgene expression. (A) Dynamin is required for clathrin-coated vesicle formation (reviewed in Warnock and Schmid, 1996; Schmid et al., 1998). GDP-bound dynamin is targeted to coated pits, where initially it is distributed evenly on the clathrin lattice. GTP/GDP exchange triggers redistribution of dynamin into collars around the necks of invaginated coated pits. GTP hydrolysis drives a second conformational change, which is required for constriction and detachment. The *K44A* mutant form of dynamin is defective in GTP binding and hydrolysis, resulting in formation of pits that are invaginated but not constricted (van der Bliek et al., 1993). (B) *prd* is expressed in a 5-cell-wide domain in odd-numbered abdominal segments (Yoffe et al., 1995). This domain includes two rows of cells anterior to the *wg*-expressing row of cells, the *wg*-expressing cells and the two rows of *en*-expressing cells posterior to the *wg* cells.

cells (Yoffe et al., 1995). Because the *prd-Gal4* is expressed in alternate segments, it provides an internal wild-type control in even-numbered segments.

In embryos where moderate *shi*^{D} expression is driven by *prd-Gal4*, Wg antibody staining in odd-numbered segments becomes concentrated in and around the *wg*-expressing cells and the staining appears less broadly diffuse than the wild-type (Fig. 2A,C). This staining pattern resembles that observed for mutant forms of Wg that are defective in movement (Dierick and Bejsovec, 1998). At higher magnification, Wg antibody staining in the affected segments appears less vesicular than that of even-numbered unaffected segments, or all segments of wild-type embryos (Fig. 2B,D). Instead it appears to accumulate predominantly around the periphery of *wg*-expressing cells. Occasionally, vesicles can be observed within the *prd* expression domain, indicating that moderate expression of *shi*^{D} reduces, but does not completely abolish, endocytosis.

This Wg protein accumulation correlates with a restricted domain of stabilized Arm. In embryos expressing *prd-Gal4*-driven *shi*^{D}, Arm stripes in odd-numbered segments appear narrower in width (Fig. 2E,G). Again, this staining pattern resembles that observed in embryos mutant for transport-defective alleles of *wg* (Dierick and Bejsovec, 1998). At higher magnification, stabilized Arm is detected over roughly 4-6 cell diameters in affected segments, as opposed to 5-8 cell diameters in unaffected segments, or in all abdominal segments of wild-type embryos (Fig. 2F,H). This range of apparent Wg action is slightly broader than is observed for *shi* loss of function, where Arm stabilization is limited to 2-4 cell diameters (Bejsovec and Wieschaus, 1995). Thus, expressing the *shi*^{D} transgene at moderate levels partly, but not completely, mimics the effects of *shi* loss of function on Wg distribution.

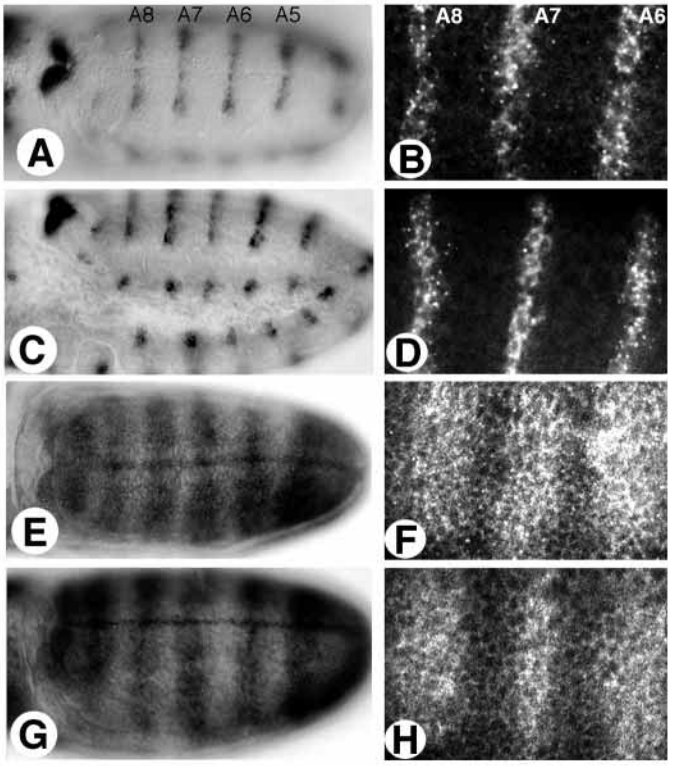


Fig. 2. Perturbing endocytosis restricts Wg protein distribution and signaling activity. (A) Wg antibody detection in a wild-type stage 10 embryo shows diffuse staining in and around the *wg*-expressing row of cells in each segment. (B) Higher magnification confocal microscopy reveals a punctate staining pattern, consistent with a vesicular localization for Wg. (C) When *shi*^{D} is expressed with the *prd-Gal4* driver line, Wg protein in odd-numbered abdominal segments shows high-level accumulation and restriction to the *wg*-expressing row of cells. (D) This corresponds with a reduction in the vesicular Wg staining and an increase in Wg accumulation at the periphery of the *wg*-expressing cells. (E) In wild-type stage 10 embryos, Arm protein appears in broad stripes centered over the *wg*-expressing row of cells in each segment. (F) At higher magnification, these stripes appear to range from 5 to 8 cell diameters in width. (G) In stage 10 embryos expressing *prd-Gal4*-driven *shi*^{D}, Arm stripes in odd-numbered segments become narrower. (H) Stripes in affected segments range from 4 to 6 cell diameters in width, as opposed to the normal 5 to 8 cell diameters in unaffected segments.

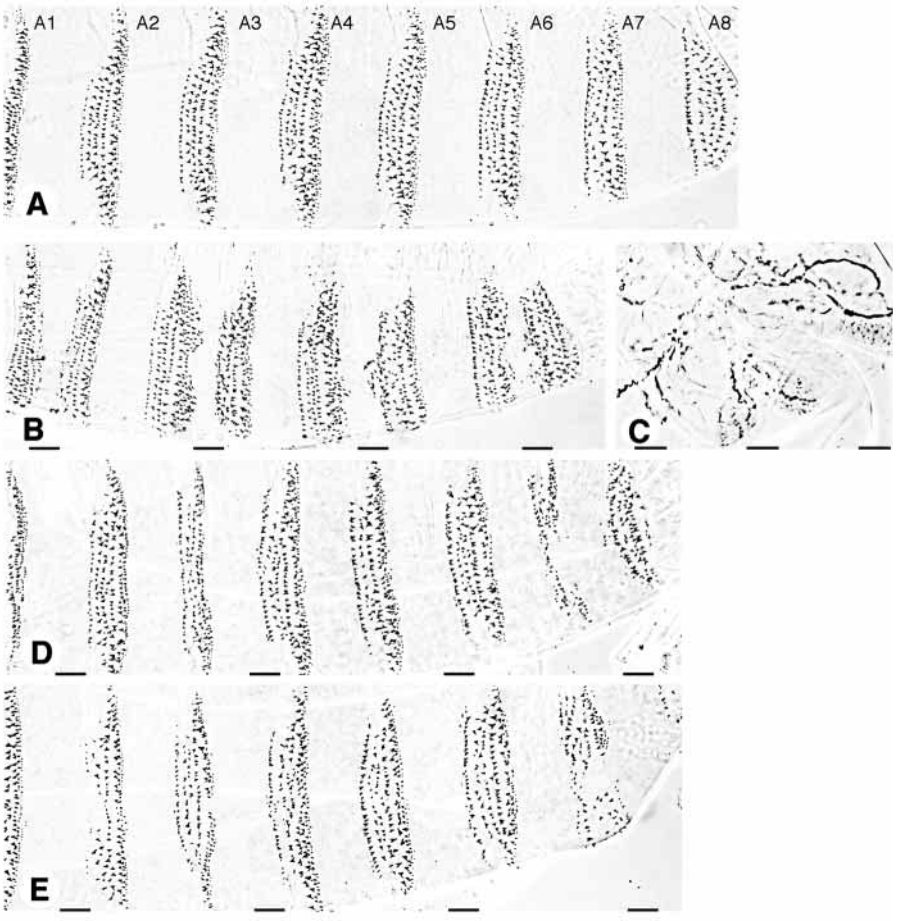


Fig. 3. Perturbing endocytosis disrupts epidermal patterning in a Wg-dependent fashion. (A) Wild-type first instar larvae display a stereotyped pattern of cuticular structures secreted by the epidermal epithelia. Six rows of denticles are arranged in a belt at the anterior of abdominal segments; each row has a characteristic size, shape and orientation. (B) *prd-Gal4*-driven expression of *UAS-shi^D* (approximate expression domain denoted by bars) causes excess denticle formation, replacing naked cuticle, in odd-numbered segments and disrupts denticle morphology in even-numbered denticle belts. (C) High-level expression of *UAS-shi^D* in the *prd* domain leads to defective cuticle deposition similar to that observed with the original *shi^{ts}* mutation. The resulting holes in the cuticle roughly correspond with the *prd* expression domain. (D) The pattern defects caused by moderate level *UAS-shi^D* expression are reversed by co-expressing *UAS-wg$^+$* in the *prd* domain. Some excess naked cuticle replaces portions of denticle belts due to ectopic activity of Wg. (E) This excess naked cuticle is also observed when *UAS-wg$^+$* alone is expressed in the *prd* domain.

Epidermal patterning is disrupted on both sides of the restricted Wg domain

shi^D interference with normal Wg movement alters cuticular patterning. Embryos expressing moderate levels of *shi^D* in the *prd* domain show a smaller expanse of naked cuticle specified in odd-numbered segments (Figs 3A,B, 4A,B). Ectopic denticles appear at the posterior edge of the odd-numbered denticle belts, replacing the portion of naked cuticle furthest away from the Wg source in that segment (Fig. 5A,B). This result suggests that during wild-type epidermal patterning, Wg moves in an anterior direction to specify the naked cuticle cell fates within each segment. Reducing endocytosis in the odd-numbered segments also causes a loss of diversity in the even-numbered abdominal denticle belts, disrupting denticle types secreted by cells posterior to the affected source of Wg (Figs 4A,B, 5A,B). These denticles are still organized into a six-row belt, but the anterior rows do not show the distinct morphologies characteristic of the wild-type denticle belt (Bejsovec and Wieschaus, 1993). This suggests that Wg protein moving in a posterior direction is responsible for directing these denticle-secreting cell fates.

We believe that the patterning defects result primarily from effects on Wg distribution and not from effects on other signaling molecules, such as Hedgehog, because we can rescue the pattern by co-expressing a *UAS-wg$^+$* transgene with the *UAS-shi^D*. In such embryos, the naked cuticle region in odd-numbered segments is restored to its normal expanse and the even-numbered denticle belts show a more normal degree of diversity (Figs 3D, 4C). In some cases, excess naked cuticle ablates a portion of the even-numbered denticle belts; this phenomenon is an expected consequence of overexpressing wild-type Wg (Noordermeer et al., 1992) and is seen when the *UAS-wg$^+$* transgene is expressed alone (Fig. 3E). Thus, providing a source of Wg coincident with the affected domain of cells restores normal patterning, indicating that transit of Wg to neighboring cells outside of the affected domain is sufficient to reverse the defect (Fig. 5C). This result demonstrates that the pattern disruptions are not a secondary consequence of *shi^D* effects on cuticle deposition, which are observed when the transgene is expressed at higher levels (Fig. 3C), and also suggests that *shi^D* expression does not significantly diminish secretion of Wg protein from the *wg*-expressing cells.

Furthermore, moderate level *shi^D* expression does not appear to block the ability of Wg to signal. Expressing *shi^D* in the *wg* domain, using the *wg-Gal4* driver, produces no deviation from the wild-type cuticular pattern (Fig. 6A). In contrast, wg-Gal4-driven expression of the dominant negative *dTCF-ΔN*, a known antagonist of Wg signaling activity (Cavallo et al., 1998), prevents naked cuticle secretion in the *wg*-expressing rows of cells (Fig. 6B). Thus the pattern defects produced by moderate levels of *shi^D* expression do not simply reflect a general antagonism of Wg function, but rather appear to result fairly specifically from reduction of Wg transport through the *shi^D*-expressing domain.

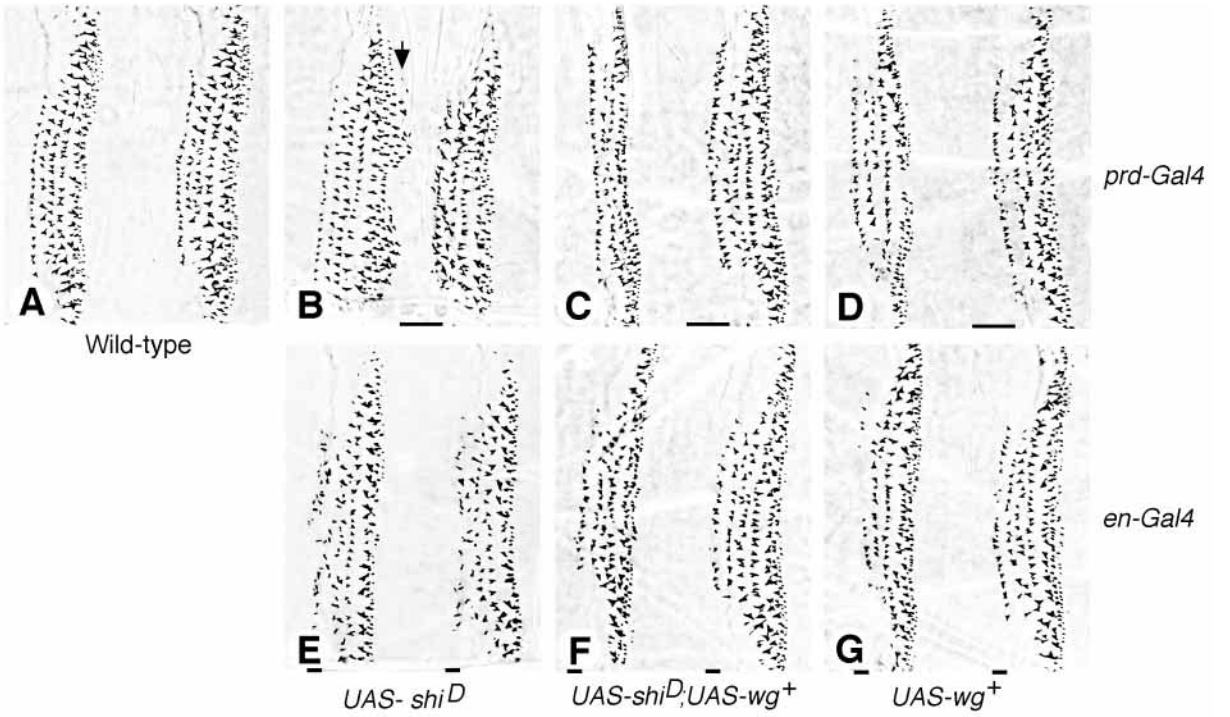


Fig. 4. Interference with Wg transport disrupts denticle identities. (A) High magnification view of abdominal segments 3(A3) and 4(A4) shows the denticle morphology characteristic for each of the six rows in the wild-type belt. (B) *shi^{D}* expressed in A3 (approximate expression domains depicted by bars) reduces the naked cuticle expanse within that segment, with ectopic denticles appearing at the posterior edge of the A3 denticle belt (arrow). *shi^{D}* expressed in A3 also causes altered morphology in the first three rows of denticles in A4. No alteration of these rows of denticles is observed in the A3 denticle belt, presumably because these denticle-secreting cells are patterned by Wg moving backward from unaffected cells in A2. (C) Both odd-numbered segment ectopic denticles, and even-numbered segment denticle morphology defects, are eliminated when wild-type *wg* is co-expressed with *shi^{D}*. Some disruption of denticle patterning at the midline is observed, but this is due to ectopic Wg activity and is observed when *UAS-wg^{+}* is expressed alone, (D). (E) *en-Gal4*-driven expression of *shi^{D}* alters denticle morphology in the first 3 rows of every denticle belt, as Wg protein transport is now affected in each segment. (F) Again, these pattern defects are reversed by co-expressing wild-type *wg* with *shi^{D}*, indicating that the defects result primarily from improper handling of Wg protein. (G) When wild-type *wg* is expressed ectopically in the *en* domain, only subtle changes to the normal denticle pattern and some excess naked cuticle are observed.

Reducing posterior movement of Wg rescues the *naked* mutant phenotype

Using *en-Gal4* to drive expression of *shi^{D}* disrupts endocytosis in the two rows of cells just posterior to the *wg*-expressing cells and effectively reduces posterior Wg movement in every segment, without affecting its anterior movement. This manipulation perturbs denticle type specification in the first few rows of denticles in each segment (Fig. 4E), similar to the pattern disruption observed in even-numbered segments when *shi^{D}* expression is driven with the *prd-Gal4* (Fig. 4B). Again, this pattern defect is reversed when *wg^{+}* is co-expressed with *shi^{D}* (Fig. 4F). These embryos show only subtle denticle type aberrations due to the ectopic expression of wild-type *wg* in the *en* domain (Fig. 4G). In contrast to the *prd-Gal4* situation, however, specification of naked cuticle within each segment is normal. This further supports the idea that anterior movement of Wg specifies the extent of naked cuticle secretion.

We find that using *en-Gal4*-driven *shi^{D}* expression to reduce posterior movement of Wg suppresses the phenotype of the segment polarity mutation, *naked (nkd)*. *nkd* mutant embryos secrete denticle belts that have essentially normal denticle type diversity but that are replaced to varying degrees by naked cuticle (Fig. 7B; Nüsslein-Volhard and Wieschaus, 1980; Jürgens et al., 1984). This excess naked cuticle depends upon Wg activity levels. The *wg; nkd* double mutant shows no naked cuticle across the ventral region and reducing the dosage of *wg* in a *nkd* mutant restores denticle belts (Bejsovec and Wieschaus, 1993). Thus wild-type *nkd* gene function appears to be involved in limiting Wg signaling activity within the segment. Consistent with this idea, Wg target genes become ectopically expressed in *nkd* mutant embryos (Martinez Arias et al., 1988; Bejsovec and Wieschaus, 1993). The *en* expression domain expands 2-3 cell diameters during stage 9 (Fig. 7E), and an ectopic stripe of *wg* expression arises at stage 10, in the row of cells posterior to this expanded *en* domain.

The posterior expansion of *en* expression suggested that *nkd* might play a role in restricting movement of Wg protein in a posterior direction. Indeed, when we generate *nkd* mutant embryos that express *shi^{D}* at moderate levels in the *en* domain, we see a dramatic reduction in the amount of naked cuticle specified (Fig. 7B,C). These embryos are very similar in appearance to wild-type embryos in which *en-Gal4* drives *shi^{D}* expression (Figs 4E, 7A) except that the *nkd* mutant head defect is not fully rescued. *en-Gal4*-driven *shi^{D}* expression also prevents the ectopic activation of *en* expression in *nkd* mutants (Fig. 7E,F). The stripes of *en* expression in the thoracic and abdominal segments are restored to the normal width, although some expansion is still observed in the head segments (Fig. 7D,F).

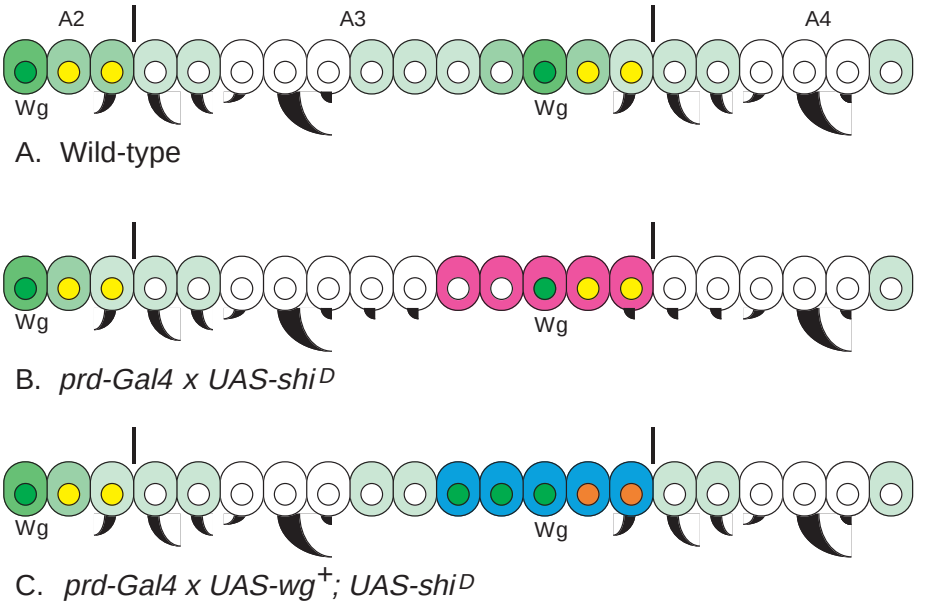


Fig. 5. Schematic diagram of the effects of Wg protein distribution on epidermal patterning. (A) During stage 9, Wg protein is distributed on either side of the *wg*-expressing row of cells (van den Heuvel et al., 1989; Gonzalez et al., 1991) and generates both aspects of cuticular pattern: anterior movement is required for specifying naked cuticle cell fate and posterior movement is required for proper patterning of the first few rows of denticles. The denticles are produced as outpocketings of the apical epidermal cell membranes, just prior to the secretion of cuticle at stage 17 (Campos-Ortega and Hartenstein, 1985). (B) Inhibiting Wg movement through cells on either side of the *wg*-expressing row of cells (in A3) disrupts both processes: ectopic denticles replace naked cuticle anterior to the affected domain (A3) and denticle diversification is diminished posterior to the affected domain (A4). (C) Co-expressing wild-type *wg* with shi^D allows normal patterning to proceed, since the ectopic *wg*-expressing cells directly contact non-shi^D-expressing cells and thus Wg can transit through the epithelium properly.

Since wild-type Wg signaling activity is required for stabilization of *en* expression (DiNardo et al., 1988; Martinez Arias et al., 1988), En stripes of normal width indicate that sufficient functional Wg contacts both rows of *en*-expressing cells to produce normal target gene regulation. Again, this result demonstrates that expression of shi^D does not interfere with Wg signal transduction and supports the idea that moderate level shi^D expression reduces, but does not eliminate, transport of Wg across the affected domain. In contrast, embryos expressing high level shi^D in the *en* domain show a narrowed stripe of En antibody staining, suggesting that Wg can no longer traverse the first row of *en*-expressing cells to stabilize *en* in the second row (Fig. 7G,H). However, because of the severe effects of a more complete endocytotic block, these embryos do not secrete cuticle properly and so the effects on cuticle pattern are not interpretable (Fig. 7I).

Direction of Wg protein movement changes during development

During early stages of wild-type embryogenesis, Wg protein can be detected at high levels in cells both anterior and posterior to the *wg*-expressing row of cells (Figs 8A, 9A; van

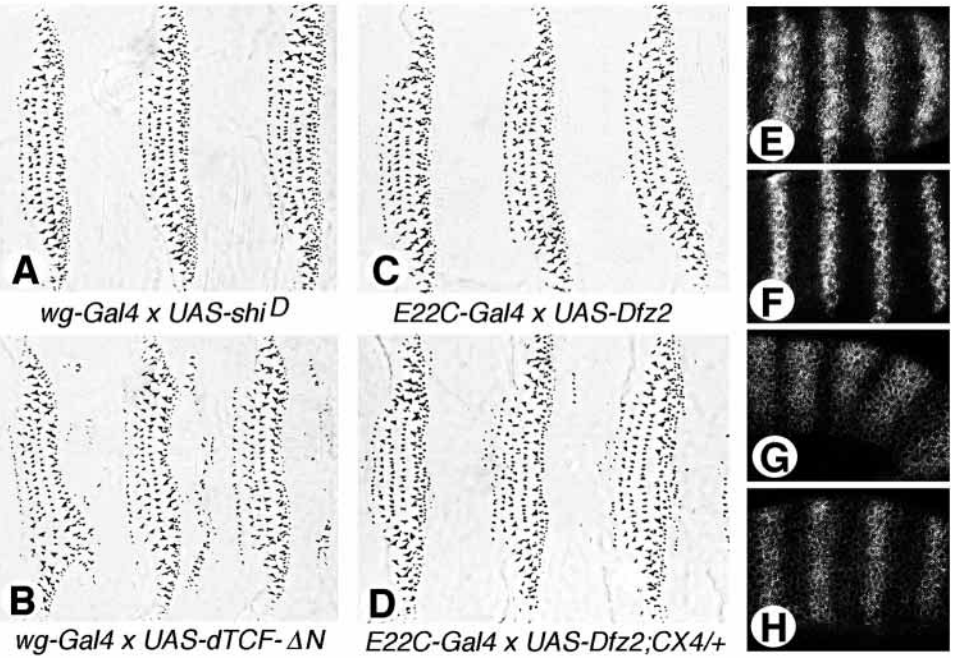


Fig. 6. shi^D expression does not disrupt Wg signaling and its effects on Wg are mimicked by overexpressing Wg receptor. (A) When shi^D expression is driven in the *wg* domain, no alteration from the wild-type cuticle pattern is observed (compare Fig. 3A). 99% of these animals hatch and survive to adulthood (n=239). (B) In contrast, expressing dominant negative dTCF in the *wg* domain antagonizes Wg signaling in the *wg*-expressing row of cells and blocks the specification of naked cuticle cell fate. (C) Ubiquitous overexpression of the Wg receptor, Dfz2, does not alter epidermal patterning in a wild-type background (n=302). (D) When *Dfz2* overexpression is driven in wg^{CX4} heterozygotes, mild patterning defects are observed. 30% of the cross-progeny from wg^{CX4}/*CyO;UAS-Dfz2* (homozygous) × *E22C-Gal4* (homozygous) show this phenotype (n=344), we infer that this represents 60% of the *wg* heterozygotes. Overexpressing *Dfz2* in a wg^{CX4} heterozygous background also narrows the width of Wg stripes (F), and Arm stripes (H), compared with those of non-transgenic heterozygotes (E, G, respectively).

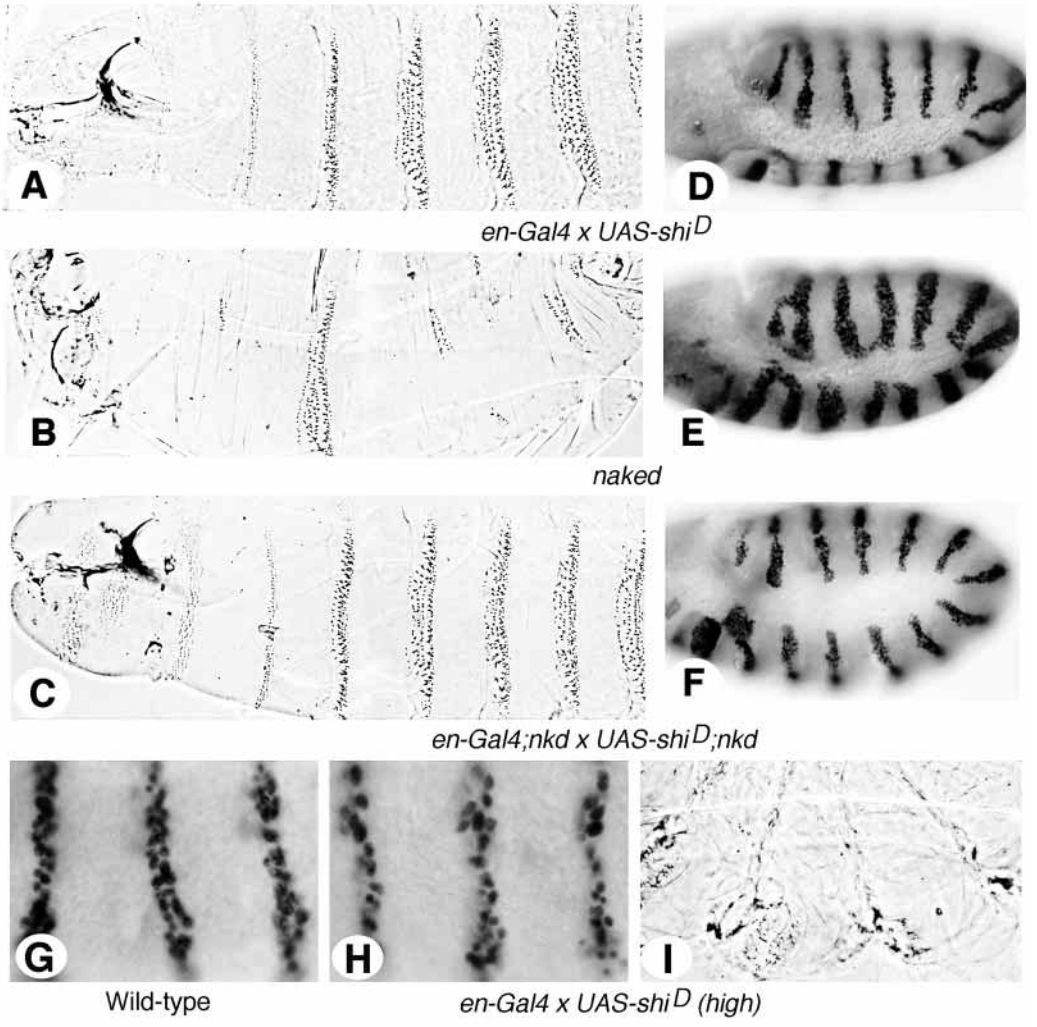


Fig. 7. Restricting posterior movement of Wg rescues the *nkd* mutant phenotype. (A) *en-Gal4*-driven expression of shi^D produces pattern disruptions in the segmental denticle belts, but does not disrupt head development. (B) *nkd* mutant embryos show excess naked cuticle replacing denticle belts in most segments, and also display severe head defects. (C) *en-Gal4*-driven shi^D suppresses the excess naked cuticle formation in *nkd* homozygous embryos, and partially, but not completely, rescues the head defect. (D) The *en* expression pattern is not altered by *en-Gal4*-driven expression of shi^D in wild-type embryos. (E) *nkd* mutant embryos show expansion of the *en* expression domain. (F) This expansion is rescued in the thoracic and abdominal segments by *en-Gal4*-driven expression of shi^D. Note that some expansion is still observed in head segments, allowing easy identification of the *nkd* homozygous embryos. When shi^D is expressed at high level in the *en* domain, the number of cells in which En protein can be detected is reduced (H), relative to wild-type (G). This presumably reflects a more complete block to endocytosis, preventing Wg from moving to the second row of *en*-expressing cells, and is associated with severe segmental defects in cuticle deposition (I).

den Heuvel et al., 1989; Gonzalez et al., 1991). Diversity of denticle types, as well as stabilization of *en* expression in the adjacent cells, are specified by Wg activity during these early stages of embryonic development (Bejsovec and Martinez-Arias, 1991). By mid-stage 10 (Figs 8B, 9B), when Wg is no longer required for denticle specification or *en* stabilization, the Wg protein distribution shifts and Wg appears to be excluded from the *en*-expressing cells (Gonzalez et al., 1991; Bejsovec and Martinez-Arias, 1991). This exclusion is not observed in *nkd* mutants at the same stage (Fig. 8C). Rather, Wg protein continues to be detected in cells on either side of the *wg*-expressing row of cells and the levels become substantially higher due to the ectopic stripe of *wg* expression (Fig. 9C). These results suggest that *nkd* gene function may play a role in the posterior restriction of Wg protein that occurs during stage 10. Hence the mutant phenotype is rescued dramatically when we produce this restriction artificially, by expressing shi^D in the *en*-expressing row of cells (Fig. 7C,F). All stage 11 and 12 embryos derived from this cross show posterior restriction of Wg protein, indicating that the *nkd* homozygotes do not exhibit excess posterior movement of Wg under these conditions (n>100).

We suspect that, in wild-type embryos, this restrictive function is not limited to the *en*-expressing cells. If this were the case, then we would expect to observe excess naked cuticle replacing denticle belts when wg^+ is expressed in the *en* domain. Instead, *en-Gal4*-driven wg^+ either alone (Fig. 4G) or when co-expressed with shi^D (Fig. 4F), does not produce substantial amounts of ectopic naked cuticle. Thus, it seems likely that some ability to restrict posterior Wg movement during later stages is shared by the rows of cells at the anterior of each segment.

Overexpressing Dfz2 restricts Wg protein distribution

We believe that our analyses of Wg transport by perturbing endocytosis are physiologically relevant because we can produce a similar inhibition of transport by overexpressing the cognate receptor for Wg, Dfz2 (Bhanot et al., 1996; Cadigan et al., 1998). We presume that these effects result from sequestering ligand, because we observe pattern defects only when Wg levels are limiting. We detect no change from the wild-type cuticle pattern when *Dfz2* is driven at ubiquitous high levels of expression with *E22C-Gal4* (Fig. 6C). However, in embryos heterozygous for a null mutation of *wg*, we observe significant pattern defects at a frequency of 60% (Fig. 6D). Ectopic denticles appear in the domain of cells that normally secrete naked cuticle, similar to what is observed in segments

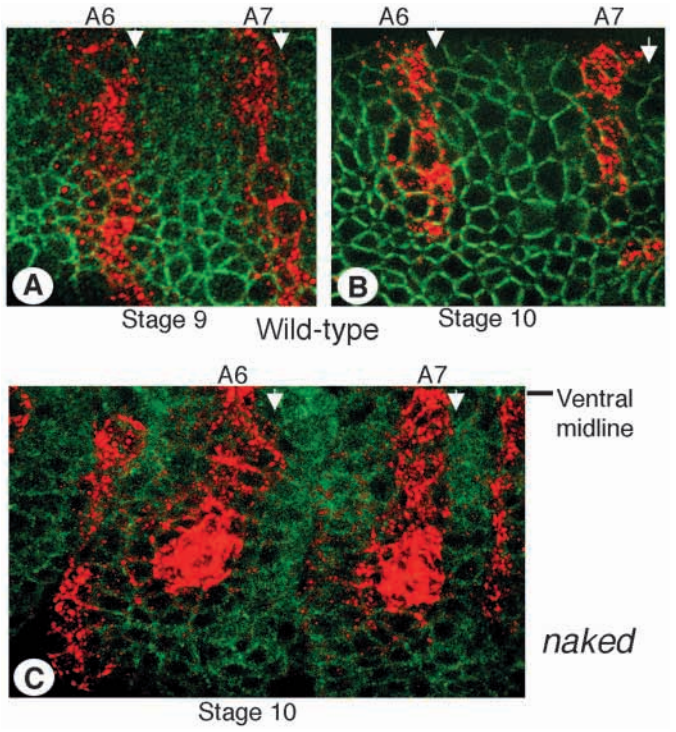


Fig. 8. Wg protein shows a posterior restriction in late stages of wild-type development. (A) Confocal microscopy of two abdominal segments in a wild-type stage 9 embryo, doubly labelled for Wg in red and Neurotactin in green, shows roughly equal distribution of Wg protein on either side of the *wg*-expressing rows of cells. Arrows mark the approximate position of the rows of *en*-expressing cells in this and subsequent panels (see also Gonzalez et al., 1991). (B) In a wild-type stage 10 embryo, Wg appears to be excluded from cells posterior to the *wg*-expressing cells (see also Bejsovec and Martinez Arias, 1991; Gonzalez et al., 1991). (C) This exclusion is not observed in stage 10 *nkd* mutant embryos. These embryos show higher levels of Wg across the segment, in part because of the induction of an ectopic stripe of *wg* expression several rows of cells anterior to the endogenous stripe. An abnormally deep ectopic segmental furrow forms between this ectopic *wg* stripe and the expanded *en* expression domain just anterior to it (Bejsovec and Wieschaus, 1993).

where anterior Wg transport is perturbed by shi^D (Fig. 3B). These pattern defects caused by *Dfz2* overexpression are accompanied by a restricted Wg protein distribution and by a narrowed domain of Arm stabilization (Fig. 6E-H). However, it is not possible to directly compare *Dfz2* with shi^D in this experiment. *E22C-Gal4*-driven expression of shi^D, even with *UAS* lines that express at low levels, results in cell death and failure to secrete cuticle as was the case with the original shi^{ts} mutation at restrictive temperature.

DISCUSSION

Our results indicate that dynamic changes in Wg protein distribution across the segment are functionally important for the temporally and spatially precise specification of cell fate decisions. Reducing endocytosis in defined domains within the segment, through moderate-level expression of a dominant negative form of *shibire*, alters the normal distribution of Wg and changes the domain of cells that respond to Wg. When expressed using the *prd-Gal4*, shi^D reduces both anterior and posterior movement of Wg protein, causing it to accumulate in and around the *wg*-expressing row of cells. Driving expression of shi^D with the *en-Gal4* reduces movement only in the posterior direction, since the *en*-expressing cells are a non-overlapping cell population just posterior to the *wg*-expressing row of cells (DiNardo et al., 1988; Dougan and DiNardo, 1992).

The effects on cuticular pattern elements indicate that Wg moving in an anterior direction from the row of *wg*-expressing cells defines the domain of cells destined to secrete naked cuticle, whereas posterior movement of Wg is required for correct specification of denticle types in the anterior of the adjacent segment. The patterning defects caused by shi^D expression are reversed by co-expression with wg^+, suggesting that the primary effect of reducing endocytosis in the embryonic epidermis is a disruption of Wg protein transport.

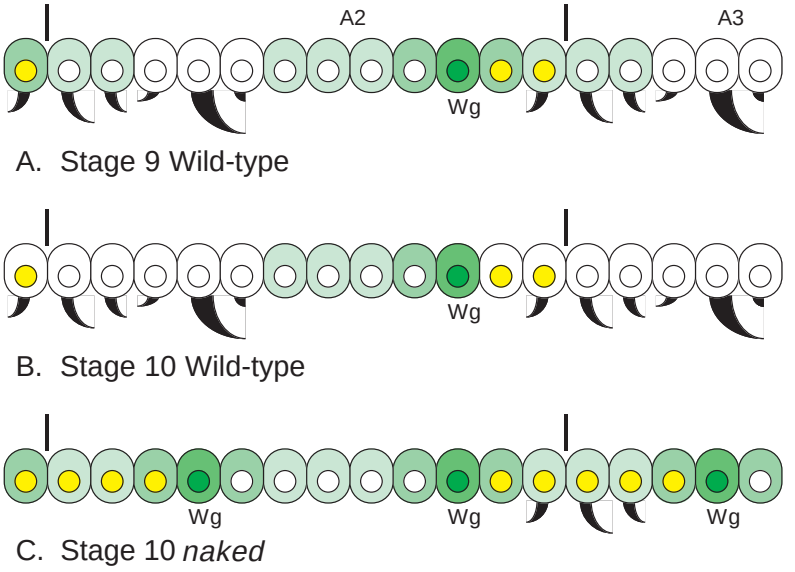


Fig. 9. Schematic diagram of Wg protein distribution in wild-type and *nkd* mutant embryos. (A) Wg protein distribution extends both anterior and posterior to the source row of cells in wild-type stage 9 embryos. Thus Wg contacts cells lying posterior to the *wg* domain and transmits positional information that will influence denticle morphology at later stages. (B) During stage 10, Wg is no longer detected in the cells lying posterior to the *wg*-expressing cells. (C) *nkd* mutants do not show this posterior restriction, *en* expression expands in a posterior direction and an ectopic stripe of *wg*-expressing cells is induced. The resulting high level Wg signaling at late stages causes re-specification of cells that would normally secrete denticles to instead secrete naked cuticle. This suggests that wild-type *nkd* gene function may be involved in changing the direction of Wg protein transport, thereby protecting denticle-secreting cells from exposure to Wg signal at late stages.

Moreover, *en-Gal4*-driven *shi*^{D} reduces endocytosis in a non-*wg*-expressing group of cells, and causes patterning defects in the cell population posterior to the *en* domain. Thus, reducing Wg transit through the *en* cells 'casts a shadow', producing patterning anomalies in an otherwise wild-type cell population. This supports the idea that Wg ligand is moved by active cellular processes through cells to arrive at distant target cell populations in the embryo.

Furthermore, our results suggest that, during normal development, the temporal changes observed in directionality of Wg protein movement (Gonzalez et al., 1991) may correlate with the temporal changes in its apparent function (Bejsovec and Martinez-Arias, 1991). In wild-type embryos prior to stage 10, Wg protein is detected over many cell diameters both anterior and posterior to the *wg*-expressing row of cells (van den Heuvel et al., 1989; Gonzalez et al., 1991). We show here that disrupting posterior movement of Wg alters patterning of at least the first three rows of denticles in the segment posterior to the affected source of Wg. Thus, posterior movement of Wg is detectable during the early time period when Wg activity is required in these cells for the generation of diverse denticle types and for the stabilization of *en* expression (Bejsovec and Martinez-Arias, 1991).

At and after stage 10, Wg protein is no longer detected in cells posterior to the *wg*-expressing row, including the *en*-expressing cells of that segment, and shows an asymmetric distribution toward the anterior of the segment (Bejsovec and Martinez-Arias, 1991; Gonzalez et al., 1991). Our results correlate this anterior movement with specification of the correct expanse of naked cuticle-secreting cells, presumably through Wg-mediated antagonism of the EGF pathway (O'Keefe et al., 1997; Szuts et al., 1997). This is consistent with previous reports that, after stage 10, Wg is no longer required for maintenance of *en* expression (Bejsovec and Martinez-Arias, 1991; Heemskerk et al., 1991) or for the generation of denticle diversity, and instead promotes specification of naked cuticle cell fate (Bejsovec and Martinez-Arias, 1991).

It is unclear by what mechanism Wg is excluded from the posterior cells at stage 10. We propose that wild-type *nkd* gene function may contribute to the change in direction of Wg protein movement. Reducing Wg movement through the *en*-expressing cells eliminates Wg-mediated specification of excess naked cuticle and substantially rescues the *nkd* mutant phenotype. Thus, posterior movement of Wg from the adjacent segment, and not anterior movement of Wg within the segment, appears to be responsible for the *naked* mutant phenotype. This observation suggests a role for *nkd*$^{+}$ gene function in restricting posterior Wg transport.

Although some aspects of Wg transport appear to be independent of Wg signal transduction (Bejsovec and Wieschaus, 1995; Hays et al., 1997; Dierick and Bejsovec, 1998), the two processes cannot be completely separated. Overexpression of Dfz2, a Wg signaling receptor, appears to restrict the distribution of the Wg protein, suggesting that it has the capacity to sequester ligand. In contrast, Dfz2 overexpression in the imaginal disc has been shown to enhance the transport of Wg protein and consequently increase its range of activity (Cadigan et al., 1998). This dramatic change in the role of Dfz2 from embryo to imaginal disc suggests that mechanisms controlling Wg distribution may differ between these two developmental stages of *Drosophila*. For example, recent work has revealed that imaginal disc cells project cytoplasmic extensions, called cytonemes, toward the source of signaling molecules at the center of the discs (Ramirez-Weber and Kornberg, 1999). These extensions may assist in the broad distribution and long-range activity documented for Wg in the imaginal discs (Zecca et al., 1996; Neumann and Cohen, 1997).

Such cytoplasmic extensions have not been detected in vivo in embryonic epidermal cells. Our work suggests that, if embryonic cells do produce cytonemes, they may not be functionally relevant to the distribution of Wg signaling activity. Reducing endocytosis in the two rows of *en*-expressing cells produces Wg-related pattern disruptions in the cells posterior to the affected domain. This suggests that Wg must physically move through the *en* cells in order to influence cell fate decisions in the posterior cell population. Such an effect would not be predicted if the posterior population were able to extend cytoplasmic projections through the affected 2 cell diameters and directly contact the cells expressing *wg*.

Finally, other work has shown that mutant Wg molecules that are secreted properly, but fail to signal, are transported as if by default (Bejsovec and Wieschaus, 1995). Initially, these mutant embryos show a wild-type distribution of Wg protein, but over time they begin to accumulate Wg-containing vesicles in tissues that do not express the gene and in which the protein is not normally detected. This indicates that most, if not all, embryonic cells have the ability to internalize Wg, and that this process does not require signal transduction. Moreover, it suggests that the mutant Wg ligand is able to bind to a cell surface receptor that does not transduce signal. This is consistent with a multiple-receptor model for Wg, where some Wg-binding receptors are dedicated exclusively to the transport process. Thus the dynamic distribution of Wg during development may reflect an interplay between signaling receptors and other cell surface molecules essential for ligand transport.

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