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Dual functions of *wingless* in the *Drosophila* leg imaginal disc

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SUMMARY

The *Drosophila* gene *wingless* is a member of the *Wnt* gene family, a group of genes that are involved in embryonic development and the regulation of cell proliferation. *wingless* encodes a secreted glycoprotein that plays a role in embryogenesis as well as in the development of adult structures. In the primordia of the adult limbs, the imaginal discs, *wingless* is expressed in an anterior ventral sector and is required for specification of ventral fate. Ectopic expression of low levels of Wingless in the leg discs leads to partial ventralization and outgrowths of the proximodistal axis. Wingless has thus been proposed to specify ventral fate in a concentration dependent manner (i.e., as a morphogen) and to organize the proximodistal axis. We have extended the analysis of Wingless function in the leg primordium through targeted ectopic expression. We find that Wingless has two functions in the leg disc. In the specification of ventral fate, our data indicate that Wingless does not function as a morphogen but instead appears to collaborate with other factors. In addition to its role in ventral fate specification, Wingless inhibits the commitment of dorsal cells toward a determined state and influences the regulation of proliferation. We propose a model in which Wingless achieves separate functions via spatially regulated mechanisms and discuss the significance of these functions during axial patterning and organization.

Key words: Wingless, leg disc, ventralization, plasticity, proliferation

INTRODUCTION

The *Drosophila* gene *wingless* (*wg*) encodes a secreted glycoprotein (van den Heuvel et al., 1989; Gonzalez et al., 1991) that has been shown to play a critical role in several aspects of embryogenesis (see Siegfried and Perrimon, 1994 for review). It is a member of a large group of evolutionarily conserved genes, the Wnt family, which are involved in both embryonic development and the regulation of cell proliferation (see Nusse and Varmus, 1992 and McMahon, 1992 for reviews). The analysis of *wg* in the *Drosophila* embryo has furthered our understanding of the signaling pathway of which *wg* is a part (Peifer and Bejsovec, 1992; Martinez Arias, 1993; Siegfried and Perrimon, 1994). However, details of how tissues are patterned and regulated by *wg* are better studied using the primordia of the adult structures, the imaginal discs. Unlike the embryo, where pattern is specified in two dimensions (anterioposterior, A/P, and dorsoventral, D/V) with only a few cell divisions, pattern and proliferation are coordinated in the discs. In addition, pattern formation in the limbs involves the added complexity of a third, proximodistal (P/D), axis.

Data from a number of laboratories have shown that *wg* is involved in patterning both the D/V and P/D axes. The function of *wg* in axial patterning of the limbs has been studied most extensively in the leg disc because the function of *wg* in this disc appears to be constant throughout larval development, while its function in the wing is apparently more complex (Couso et al., 1993). Loss-of-function analyses have shown that *wg* is required for specification of ventral structures, and that loss of *wg* activity can result in supernumerary limbs that result from bifurcations along the P/D axis (Baker, 1988; Peifer et al., 1990; Couso et al., 1993). Ectopic expression of *wg* in clones has provided additional insight into *wg* function in axial patterning of the leg discs. First, *wg* was shown to be sufficient for specification of a subset of ventral structures (Struhl and Basler, 1993); additional ventral lateral structures were observed as a result of ectopic *wg*, but the ventral-most structures were not. In these experiments, Struhl and Basler proposed that the lack of complete ventralization of imaginal disc derivatives was due to expression of low levels of *wg*. Wg was therefore proposed to function in a concentration dependent manner, with the ventral-most structures being the result of high levels of *wg* activity. Second, the clones of ectopic *wg* in the dorsal part of the disc resulted in supernumerary limbs. Further analysis of clones of ectopic *wg* suggested that an interaction between *wg* and *decapentaplegic (dpp)*, a *Drosophila* homolog of TGFβ (Padgett et al., 1987), leads to the specification of distal structures, around which a new P/D axis forms (Campbell et al., 1993). However, bifurcated limbs form in the absence of *wg* activity (Baker, 1988; Couso et al., 1993), indicating that *wg* is not absolutely required for generation of supernumerary axes.

Two general issues regarding *wg* function in the leg disc follow from these experiments. (1) To what extent can *wg* ventralize this tissue? Does *wg* act in a concentration-dependent manner, or are other signals required for complete ventraliza-

tion? (2) What causes outgrowths of the P/D axis to occur and what role does *wg* play in that process? To analyze the role *wg* plays during adult pattern formation, we have targeted *wg* expression to various regions of the leg imaginal disc via a GAL4-mediated ectopic expression system (Brand and Perrimon, 1993). This system provides an advantage over ectopic expression in randomly generated clones; since GAL4 driven expression is reproducible, phenotypes can be related to a known pattern of expression.

The central conclusion of our ectopic expression experiments is that Wg has two distinct, spatially regulated functions in the leg disc. In ventral cells, Wg is sufficient for partial ventralization, but we demonstrate that *wg* does not act as a morphogen in the specification of ventral fate. Its ability to ventralize cells is restricted to cells in the ventral and anterior dorsal regions. However, loss of the serine/threonine protein kinase (Siegfried et al., 1990; Bourouis et al., 1990) Zeste-white 3 (Zw3), which mediates *wg* signaling in the embryo (Siegfried et al., 1992) and in the discs (Couso et al., 1994; Diaz-Benjumea and Cohen, 1994), results in complete ventralization and is not spatially restricted, indicating that all cells can be ventralized. Zw3 appears to mediate the ventralizing effects of *wg* and also to integrate additional signals that enhance the effect of *wg* alone. In dorsal cells, we provide evidence that *wg* interferes with differentiation and influences proliferation. We discuss the relevance of these findings to patterning in the D/V and P/D axes and propose a model in which *wg* achieves its functions in the leg disc through separate, spatially regulated mechanisms.

MATERIALS AND METHODS

Generation of UASwg^{ts} flies

The UASwg^{ts} construct consists of pUAST (Brand and Perrimon, 1993) containing the wg^{ts} cDNA and an FRT cassette (R. Holmgren, unpublished; see Fig. 1A). The wg^{ts} cDNA was generated through PCR-directed mutation of the wild-type cDNA. An antisense mutagenic oligonucleotide, 5′GCCC**CTCGA-G**AAGTTTCTCGTCGAGCTGTTCCAGCGGCGATT3′ changes an A to a T (underlined), resulting in a conversion of the cysteine at position 104 to a serine. This confers temperature sensitivity to the Wg protein (van den Heuvel et al., 1993). The oligo encompasses a *Xho*I site (bold) that was used as the 3′ cloning site for the PCR-generated fragment. The 5′ untranslated region of the cDNA was removed to avoid potential regulatory elements in that region. This was accomplished with the 5′ PCR oligo: 5′CGCG**TCTAGA**C-CCGATCAGCAATAATGG3′.

An *Xba*I site (bold) was introduced 5′ to the starting ATG (underlined). The PCR fragment was cut with *Xba*I and *Xho*I and inserted back into the remainder of the *wg* cDNA. Sequencing confirmed that no mutations other than the intentional one were introduced by the PCR procedure. The FRT cassette consists of two FRT sites flanking the hsp70 polyadenylation signal. The construct was integrated into the *y w* genome via P element mediated transformation as described in Spradling (1986).

Once the construct was stably integrated, the FRT cassette was removed. To excise the FRT cassette, females of the genotype *y w/y w; UAS-FRT-*wg^{ts}*/UAS-FRT-*wg^{ts} were crossed to males of the genotype *w/Y;MKRS,* $hsFlp^{M99}$*/nkd,* which carry the flip recombinase under the control of the hsp70 promoter (Golic, 1991; Chou and Perrimon, 1992). Their progeny were heat shocked for a 2-hour period as larvae. Twenty strains were established from individual adults of the genotype *y w; UAS*wg^{ts}*/+; MKRS,* $hsFlp^{M99}$/+ by crossing to *y w* flies. Germline removal of the FRT cassette was monitored by crossing flies of each strain to 1J3 GAL4 and staining the embryos for ectopic *wg* expression. Of the twenty lines, three underwent germline excision of the FRT cassette. An insertion on the third chromosome, M7-2.1, referred to as UASwg^{ts} throughout the remainder of the text, was used for all of the experiments.

GAL4 lines

We have used three GAL4 lines to direct ectopic expression of *wg* throughout much of the disc. Two of these, 1J3 and 69B, have been described (Brand and Perrimon, 1993). A third, T80, was provided by G. Technau and expresses GAL4 ubiquitously in third instar imaginal discs (not shown). Because the phenotypes produced by expressing ectopic *wg* with these lines are similar, we have further characterized the phenotype using one, 1J3. This line is a homozygous insertion at the *hairy* locus on the third chromosome. For all experiments except those in which *lacZ* markers were examined (see below), 1J3 flies were crossed to homozygous UASwg^{ts} flies so that the progeny are heterozygous for both insertions.

A recombinant line carrying both 1J3 and the UASwg^{ts} insertion M7-2.1 was generated to facilitate the examination of specific markers following the induction of ectopic *wg*. This line is balanced over TM3, *Sb* and maintained at 25°C. In these experiments, *1J3-UAS*wg^{ts}*/TM3* females were crossed to H15 or bab*lacZ* homozygous males, and the progeny were shifted to 16°C and examined. One half of the progeny appear wild type and one half appear mutant.

To superimpose expression of *wg* and *dpp*, we crosssed $dppGAL4^{40C.6}$*/TM6B* (Staehling-Hampton et al., 1994) or *patched-GAL4* (Speicher et al., 1994; not shown) to UASwg^{ts}, both of which gave similar results. The presence of the larval marker *Tubby* on the *TM6B* chromosome allows the identification of mutant larvae in those experiments in which *UAS*wg^{ts}*/dppGAL4* discs were examined.

Immunocytochemistry

For antibody staining of discs, discs were removed from the larvae, or larval heads were inverted, and fixed in 4% formaldehyde in PBS for 35 (Wg) or 15 (Al) minutes. They were then washed in PBS and 0.1% Triton (PT), preabsorbed with 5% goat serum, and incubated in antisera directed against Wg or Al protein (van den Heuvel et al., 1989; Campbell et al., 1993), respectively. Following washes in PT, the discs were incubated in horseradish peroxidase-conjugated secondary antibodies, washed in PT, and stained with diaminobenzidine. The Wg antiserum was preabsorbed by incubating with wild-type imaginal discs.

Examination of cuticular structures

Embryonic cuticles were prepared as described (Ashburner, 1989) and viewed by dark-field microscopy. Adult structures were dissected in 70% ethanol, incubated in 10% KOH for 10 minutes at 65°C, rinsed in water, dehydrated, and mounted in Euparal or mounted directly in Hoyer's medium.

Generation of *zw3* mutant clones

To generate *zw3* mutant clones, recombination was induced through the use of yeast FRT sites and a flip recombinase (FLP). For analysis of clones in the adult, females of genotype *y* $zw3^{M11}$ FRT^{101}*/FM7* or *y w* FRT^{101} were crossed to y^{+} ovo^{D1} FRT^{101}*/Y;* $hsFLP^{F38}$*/*$hsFLP^{F38}$ males (Chou and Perrimon, 1992). The progeny were heat shocked for 2 hours at 37°C, 66-72 hours after egg laying (AEL). The presence of *y* and *y zw3* clones was scored in animals of genotype *y w* FRT^{101}*/*y^{+} ovo^{D1} FRT^{101} ; $hsFLP^{F38}$/+ and *y* $zw3^{M11}$ FRT^{101}*/*y^{+} ovo^{D1} FRT^{101}*;* $hsFLP^{F38}$/+ respectively.

For analysis of H15 expression in mutant clones in the discs, $zw3^{M11}$ FRT^{101}*/FM7* females were crossed to males of the genotype *y w* FRT^{101}*/Y; H15/+; MKRS,* $hsFLP^{M99}$/+. The progeny were

exposed to a 2-hour heat shock to generate clones during first (24-48 hours after egg laying, AEL), second (48-72 hours AEL), or late second (66-72 hours AEL) instar. Discs from female larvae were examined at late third instar.

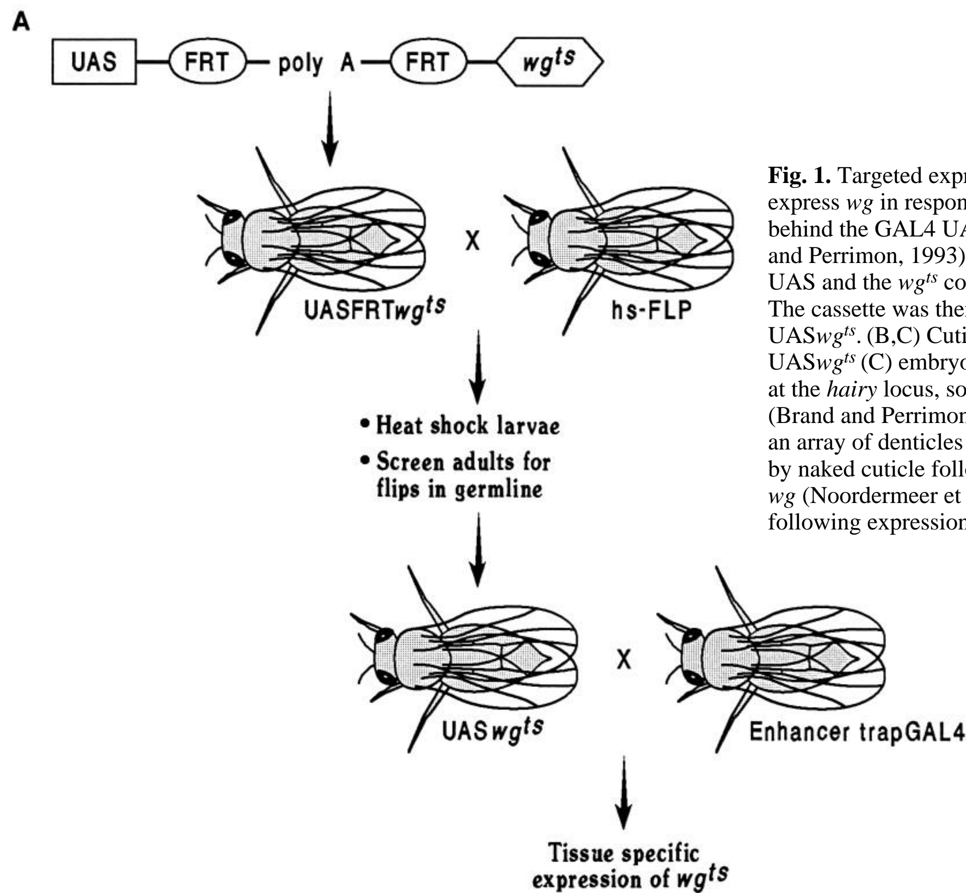
BrdU labeling of leg discs

Discs were prepared essentially as described by Usui and Kimura (1992) with the following modifications (S. Morimura, personal communication). Discs were dissected in Schneider cell medium and labeled for 15-30 minutes in 50 μg/ml BrdU in Schneider cell medium at room temperature. After rinsing in PBS, they were fixed in 3:1 ethanol:glacial acetic acid for 20 minutes, rehydrated, hydrolyzed in 2 N HCl for 30 minutes, rinsed, and preabsorbed with PBS/0.3% Triton/10% fetal calf serum. The discs were incubated with a monoclonal antibody recognizing BrdU (from Amersham) overnight at 4°C. They were rinsed in PBS/0.3% Triton, incubated with peroxidase-conjugated secondary antibody, rinsed, and stained with diaminobenzidine. Larvae were staged at late third instar (at the onset of puparium formation) by selecting those larvae that exhibited everted spiracles (Bodenstein, 1965).

RESULTS

Spatial and temporal control of *wingless* expression

To examine *wg* function in the imaginal discs, a targeted ectopic expression method was used (Brand and Perrimon, 1993) which allows control of expression both spatially and temporally. This two part system consists of the yeast transcriptional activator, GAL4, and a gene of interest, which is transcriptionally controlled by the GAL4 upstream activator sequence (UAS; see Fig. 1). To add temporal control, we introduced a mutation into the *wg* cDNA that renders the Wg product temperature sensitive (van den Heuvel et al., 1993; see Materials and Methods). Our initial attempts to transform flies



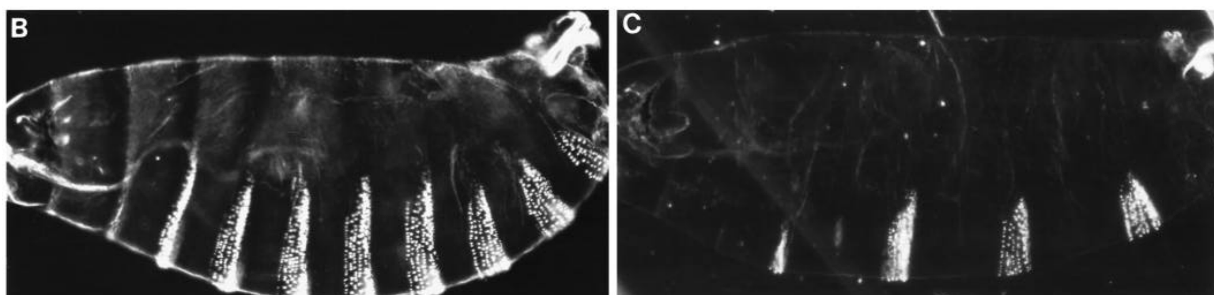


Fig. 1. Targeted expression of *wg*. (A) To generate flies that express *wg* in response to GAL4, a wg^{ts} cDNA was placed behind the GAL4 UAS in a P element-containing vector (Brand and Perrimon, 1993). An FRT cassette was inserted between the UAS and the wg^{ts} coding sequence (see Materials and Methods). The cassette was then removed to form stable lines carrying UASwg^{ts}. (B,C) Cuticular phenotype of wild-type (B) or 1J3-UASwg^{ts} (C) embryos reared at 16°C. 1J3 is a GAL4 insertion at the *hairy* locus, so GAL4 is expressed in alternate segments (Brand and Perrimon, 1993). The wild-type pattern consists of an array of denticles and naked cuticle. Denticles are replaced by naked cuticle following ubiquitous expression of wild-type *wg* (Noordermeer et al., 1992) and in alternating segments following expression of wg^{ts} in the 1J3 pattern.

with a UAS-wg^{ts} construct failed, presumably due to transient expression of *wg* following injection into embryos. To overcome this problem, a transcription terminator (R. Holmgren, unpublished data) was inserted between the UAS and the *wg* cDNA (Fig. 1A). Once genomic integration was achieved, the terminator was excised so that transcription and translation of Wg^{ts} could occur in the presence of GAL4 (see legend to Fig. 1A and Materials and Methods). By choosing among a collection of GAL4 lines and by shifting to the permissive temperature at different times, both the pattern and timing of ectopic *wg* expression can be controlled.

The activity of the ectopic Wg^{ts} was assayed in the embryonic epidermis where the effects of ectopic wild-type Wg have been characterized (Noordermeer et al., 1992). Wild-type *Drosophila* embryos exhibit a cuticular pattern on their ventral epidermis that consists of segments with hair-like denticles and naked cuticle (Fig. 1B). High levels of ubiquitous Wg in embryos results in the loss of the denticle bands, so that embryos exhibit only naked cuticle (Noordermeer et al., 1992). In the GAL4 line 1J3, GAL4 is expressed in segment-wide stripes in alternating segments (Brand and Perrimon, 1993). The expression of Wg^{ts} in this pattern at the permissive temperature of 16°C results in embryos with the cuticular pattern shown in Fig. 1C. The denticle bands in alternating segments are now reduced and replaced by naked cuticle. This phenotype is strictly dependent on the temperature, since the embryos are wild type at the restrictive temperature of 25°C and develop into viable adults (data not shown). This result demonstrates that, at the permissive temperature, the ectopic Wg^{ts} mimics wild-type Wg activity.

wg can only partially ventralize cell fates

To analyze the effects of continous ectopic *wg* expression in both dorsal and ventral cells, we initially used three GAL4 lines that direct expression to most cells in the leg discs. Since they all produced similar phenoytpes (not shown; see Materials and Methods), we concentrated on one line, 1J3 (Brand and Perrimon, 1993), for our experiments. Wg in wild-type discs is detected in ventral anterior cells (Fig. 2A). GAL4 is expressed in the 1J3 line in a broad domain that includes most of the disc except the most proximal region (Fig. 2B). UASwg^{ts} crossed to this line results in progeny (1J3-UASwg^{ts}) that express Wg in a similar pattern (Fig. 2C).

Ventral-lateral pattern is evident in the bristle pattern of the wild type male first leg (Hannah-Alava, 1958; Fig. 3A). This pattern is disrupted in the 1J3-UASwg^{ts} legs (Fig. 3B) that were shifted to 16°C during first instar: the bristles that mark the ventral-lateral surface are expanded. In addition, elements of the dorsal surface are missing, the leg circumference is increased, and the claw is often duplicated. Expansion of the ventral-lateral bristles and loss of dorsal structures occur with 100% penetrance.

The ventral-most pattern elements are clearly marked in the second leg (Hannah-Alava, 1958; Fig. 3C) and are not expanded in the presence of ectopic Wg (Fig. 3D). Thus, high levels of expression of *wg* in dorsal cells result in the expansion of the same structures that were reported to be expanded following low levels of expression of *wg* in dorsal clones (Struhl and Basler, 1993). In addition, although ectopic Wg is provided in addition to endogenous Wg in ventral cells, we do not detect an expansion of ventral-most structures, indicating that a gradient of Wg activity is not important for ventral specification.

To determine the stage at which ectopic Wg can exert its effects, we shifted 1J3-UASwg^{ts} larvae to 16°C at various stages of development. Wg can have similar effects on leg development as late as third instar: the phenotypes of legs that arise from discs exposed to ectopic Wg beginning at first, second, or third instar are comparable (not shown).

The conclusion from the cuticular phenotype that Wg can only partially ventralize cell fate is supported by the expression of the molecular marker, H15, which is expressed in ventral cells (Brook et al., 1993; Fig. 3E). This marker appears to be differentially expressed in wild-type discs, with higher expression observed in the ventral-most region of the leg disc. Only the lower level of H15 expression is expanded dorsally following ectopic expression of *wg* in the 1J3 pattern (Fig. 3F). The expression pattern of H15 also shows that Wg cannot ven-

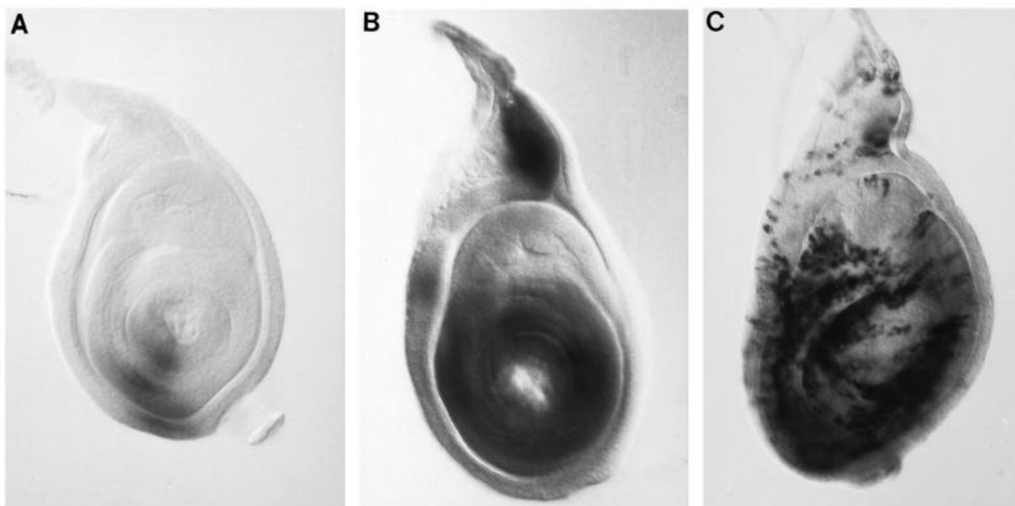


Fig. 2. Ectopic expression in 3rd instar leg discs. In all discs, anterior is left, dorsal is top. During morphogenesis, discs evert telescopically; thus, proximal elements are specified at outer edges while the center is the presumptive distal end. (A) Endogenous expression of Wg is detected in the anterior ventral region using an antibody against Wg (van den Heuvel et al., 1989). (B) GAL4 activity in the 1J3 line at 16°C is defined by staining 1J3-UAS*lacZ* discs with XGal (Brand and Perrimon, 1993). Disc expression of GAL4 has been detected in this line from mid-second instar (not shown). (C) Ectopic Wg^{ts} expression is detected in the same pattern, although the disc is misshapen as a result of ectopic Wg activity beginning at first instar. We have detected a cold sensitivity of GAL4 that results in a 5- to 10-fold decrease in activity at 16°C compared to 25°C. Therefore all of our expression analyses were performed in discs from larvae reared at 16°C.

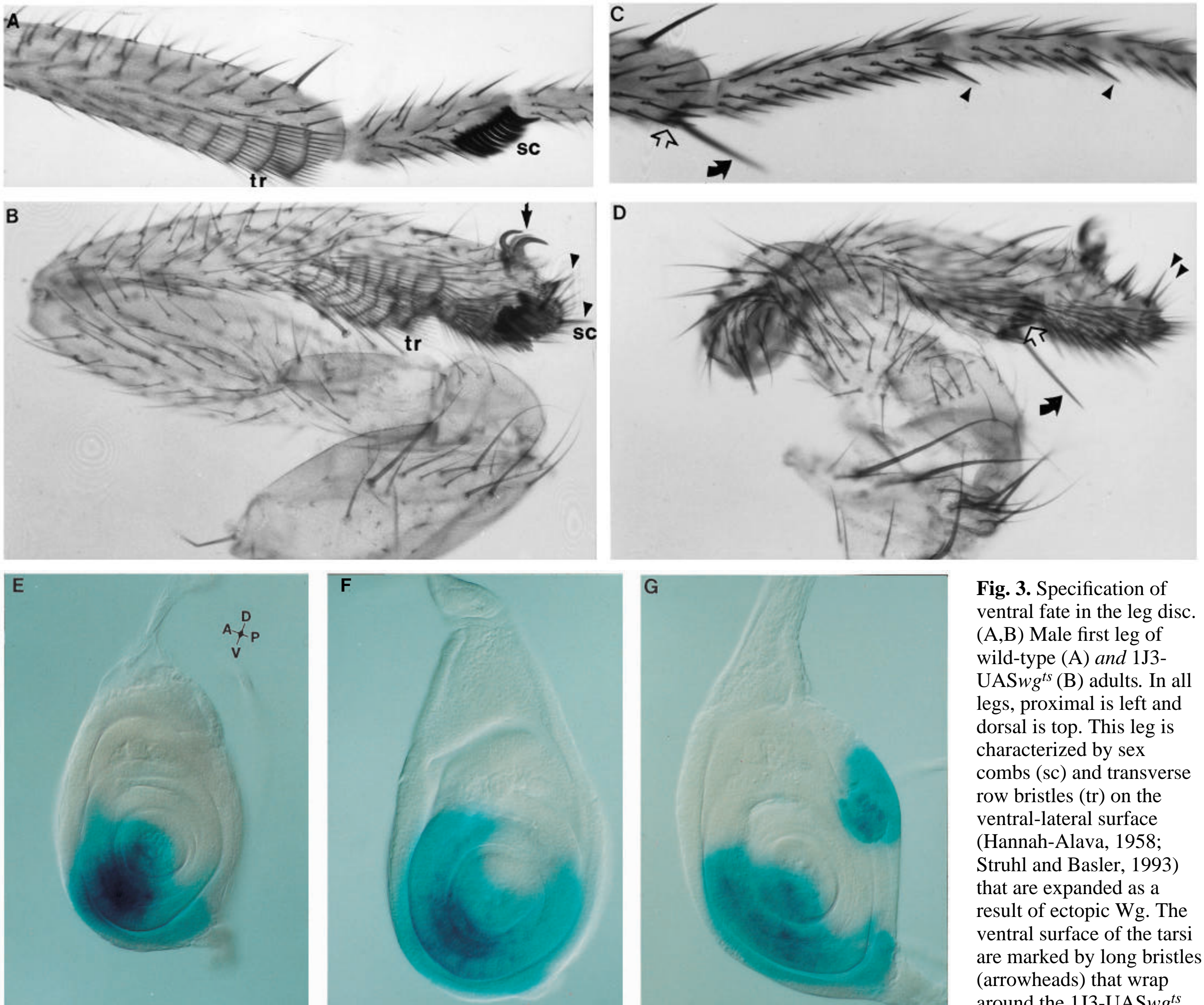


Fig. 3. Specification of ventral fate in the leg disc. (A,B) Male first leg of wild-type (A) *and* 1J3-UAS*wg*^{ts} (B) adults. In all legs, proximal is left and dorsal is top. This leg is characterized by sex combs (sc) and transverse row bristles (tr) on the ventral-lateral surface (Hannah-Alava, 1958; Struhl and Basler, 1993) that are expanded as a result of ectopic Wg. The ventral surface of the tarsi are marked by long bristles (arrowheads) that wrap around the 1J3-UAS*wg*^{ts} legs to the claw (arrow), which is often duplicated. The long dorsal bristles of the femur are often reduced in number (Hannah-Alava, 1958; not visible in this focal plane). (C,D) Second leg of wild-type (C) and 1J3-UAS*wg*^{ts} (D) adults. The ventral-most surface is marked at the tibial-tarsal joint by a long bristle (arrow) surrounded by a semi-circle of short spur bristles which extend laterally (open arrow). This surface of the tarsi is marked by 2 rows of longer thick bristles (arrowheads), one of which is out of the focal plane in C. These markers of the ventral-most surface are not expanded following ectopic Wg expression. Flies were reared at 16°C beginning at first instar. All of these individuals die as pharate adults. Variation from the shown phenotypes are observed with respect to the degree to which proximal elements are distorted. However, the legs shown here are intermediate in this range and represent the majority of those legs that have been examined. (E-G) H15 expression in wild-type (E), 1J3-UAS*wg*^{ts} (F), and *zw3*^{M11} mutant clone-containing (G), 3rd instar leg discs. H15 is a *lacZ* enhancer trap line (Brook et al., 1993), the expression of which is detected here with XGal. The less intense staining is expanded dorsally in the 1J3-UAS*wg*^{ts} discs. The darker staining remains restricted to the most ventral region of the disc. Staining is excluded from the posterior dorsal region. Loss of *zw3* however, results in H15 expression in this quadrant. Discs in E,F developed at 16°C beginning at early third instar. The clone in G was induced during first instar. Clones induced later in development (through late second instar) also exhibit ectopic H15 staining in all regions of the discs (not shown).

tralize the posterior dorsal quadrant of the leg disc, which is consistent with the lack of ventralization around the entire circumference of the adult legs. This shows that the ability of Wg to specify ventral fate is spatially restricted.

All cells in the disc are able to adopt a ventral fate

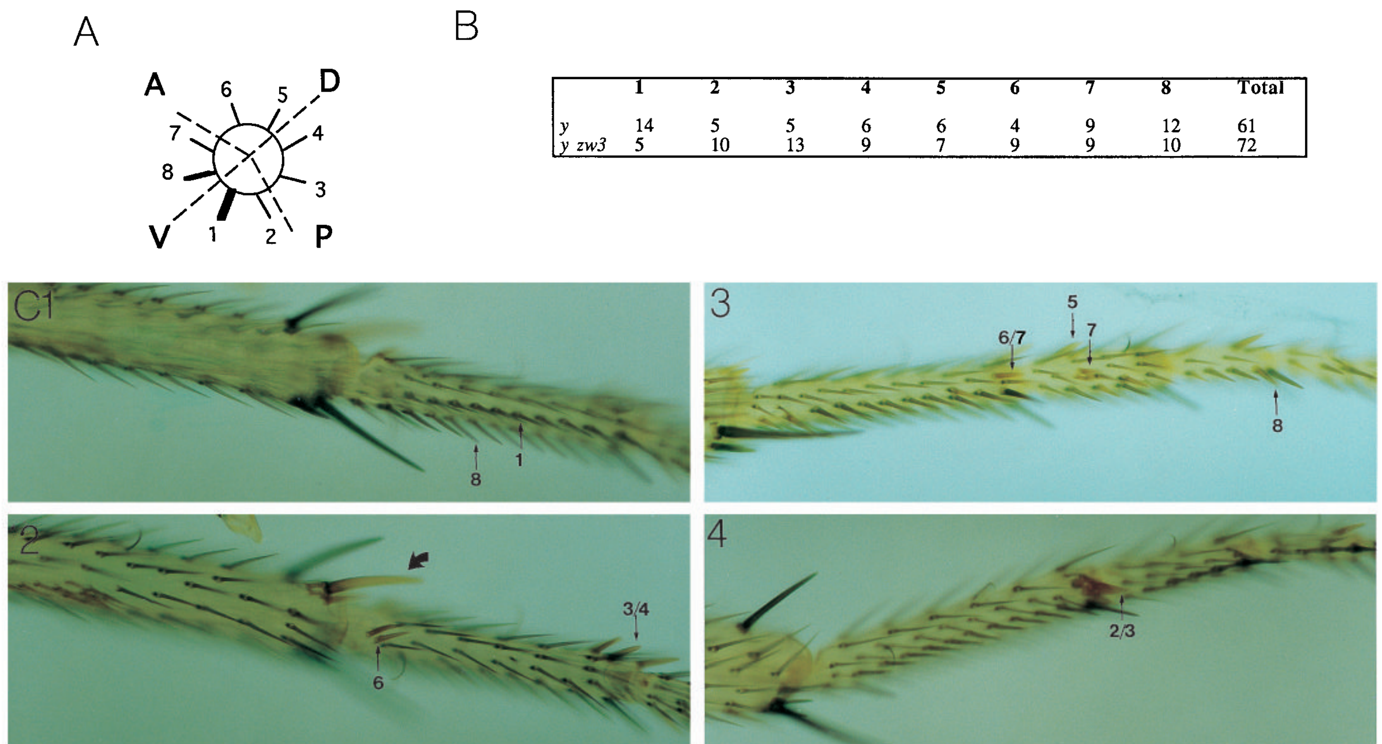
The inability of ectopic Wg to ventralize the posterior dorsal quadrant could reflect an inability of these cells to assume a ventral fate. To analyze their potential for ventralization, we examined the effects of loss of Zw3 activity. In the embryo, ectopic expression of *wg* and loss of *zw3* result in similar phenotypes (Noordermeer et al., 1992; Perrimon and Smouse, 1989), and epistasis experiments indicate that Wg signaling operates through repression of Zw3 (Siegfried et al., 1992). A similar mechanism has been shown to be used in the discs (Couso et al., 1994; Diaz-Benjumea and Cohen, 1994). In the

leg disc, loss of *zw3* results in ectopic H15 expression in dorsal posterior as well as dorsal anterior cells (Fig. 3G). This indicates that the failure of the dorsal posterior cells to be ventralized by ectopic Wg is not due to their inability to be ventralized.

The timing of ectopic *wg* expression might explain both the failure of the posterior dorsal cells to be ventralized as well as the partial ventralization of the anterior dorsal cells. Detection of GAL4 in 1J3 discs younger than mid-second instar has been hampered by strong muscle expression in this line (not shown); therefore, although GAL4 is expressed in other tissues early in larval life, disc expression has not been observed prior to mid-second instar. This may be too late for dorsal cells to be fully ventralized. To determine if this was the case, we induced *zw3* mutant clones during late second instar (66-72 hours AEL; Fig. 4). These clones give rise to fully ventralized dorsal bristles in both anterior and posterior compartments. This indicates that the restrictions in the ability of Wg to specify ventral fate is not due to late expression. All cells of the leg disc have the capacity for complete ventralization, and all signal(s) that are required for this process appear to be mediated through repression of *zw3*.

Ectopic *wg* expression interferes with dorsal differentiation

Since the posterior dorsal cells can be specified as ventral, their failure to do this in response to ectopic *wg* might be explained by their inability to receive the Wg signal. To determine whether Wg is capable of eliciting a response in this quadrant, we analyzed the expression of the gene *bric-a-brac (bab)*, which marks the determination of cells in all quadrants as tarsi (Godt et al., 1993; Fig. 5A). In 1J3-UASwg^{ts} discs shifted to 16°C during the first instar, *bab* expression in dorsal cells is disrupted (Fig. 5B): anterior dorsal cells variably activate *bab* expression while those in the dorsal posterior portion never do. A similar effect is seen on expression of *apterous (ap)*, a second gene that is expressed around the entire circumference (not shown). These cells are viable as indicated by their failure to absorb a vital dye (not shown). Thus, posterior dorsal cells can respond to Wg, although they respond differently than do cells in the ventral and anterior dorsal regions (see Fig. 8).



	1	2	3	4	5	6	7	8	Total
y	14	5	5	6	6	4	9	12	61
y zw3	5	10	13	9	7	9	9	10	72

Fig. 4. Analysis of *zw3* mutant clones induced during late second instar. (A) Diagram of a cross section through a mesothoracic (second) leg at the level of the tarsi. The A/P and D/V axes are presented at an angle that corresponds to the angle at which they lie on the fate map of the third instar disc. The A/P border is placed according to Steiner (1976), and the position of the bristle rows is from Held et al. (1994). The two most ventral rows of bristles are the thickest. (B) Table showing numbers of clones generated in each bristle row. *y* or *y zw3* clones were induced at 66-72 hours AEL, and 25 legs of each genotype were scored. *zw3* mutant clones gave rise to row 1 or 8 bristles in all the bristle rows, including rows 3 and 4 of the posterior dorsal quadrant. Bristles were scored as a clone if no y^+ bristles interrupted them. (C) Phenotypes of *y zw3* clones. C1 and C2 depict different focal planes of the same leg. In C1, y^+ bristles of rows 1 and 8 are pointed out for reference. The apical bristle of the ventral tibia is in the anterior compartment and lies closest to row 8 of the tarsi (Held et al., 1994). In the focal plane of C2, a duplicate *y* apical bristle is observed. *y zw3* clones in row 3/4 and row 6 exhibit bristles characteristic of rows 1 and 8. (C3) *y zw3* clones in rows 5, 6/7, 7, and 8 elaborate identical bristles. (C4) A *y zw3* clone in row 2/3 has been ventralized to become like row 1 or 8. The neurogenic effect of *zw3* (Simpson, 1988; Diaz-Benjumea and Cohen, 1994) produces a clump of bristles. This effect is not always observed in clones induced during late second instar, perhaps because of the small clone size. Interestingly, although clones induced earlier often produce bifurcated legs and pattern intercalation (not shown; Diaz-Benjumea and Cohen, 1994), this has not been observed when mutant clones are induced during late second instar.

One possible reason for the cells' failure to activate tarsal-specific genes is a transformation to either a more proximal or distal fate. Although the cuticular phenotype offered no evidence of a proximal transformation, the duplication of claws suggested that a distal transformation occurred (Fig. 3). To analyze this possibility molecularly, we examined the expression of *aristaless (al)*, which marks the presumptive distal end of the leg at the disc center (Campbell et al., 1993; Fig. 5C). Expression of Al is expanded in the 1J3-UASwg^{ts} leg discs, although the expansion does not appear to be sufficient to account for the loss of *bab* expression (Fig. 5D). Rather, the expression expands only along the A/P border, supporting an hypothesis that a factor in this region interacts with Wg in the induction of *al* expression (Campbell et al., 1993). The failure of many of the presumptive tarsal cells to activate tarsal gene expression suggests that Wg interferes with the transition of these cells to a committed state. The partial loss of *bab* and *ap* expression in the anterior dorsal quadrant suggests that these cells may also respond to Wg by failing to be determined, although in a less penetrant manner.

Since ectopic expression of vertebrate Wnt-1 in the limb bud results in inhibition of differentiation and aberrant regulation of cell proliferation (Zakany and Duboule, 1993), proliferation in the discs following ectopic Wg expression might also be expected to be altered. To examine this possibility, we labeled discs with the nucleotide analog, bromodeoxyuridine (BrdU), which is incorporated in the DNA of dividing cells. The pattern of incorporation in late third instar wild-type leg discs is largely random (Fig. 5E). The center of the disc appears to be quiescent. Analysis of 1J3-UASwg^{ts} discs reveals that dorsal cells overproliferate (Fig. 5F). Surprisingly, the region of increased proliferation is largely outside the region of

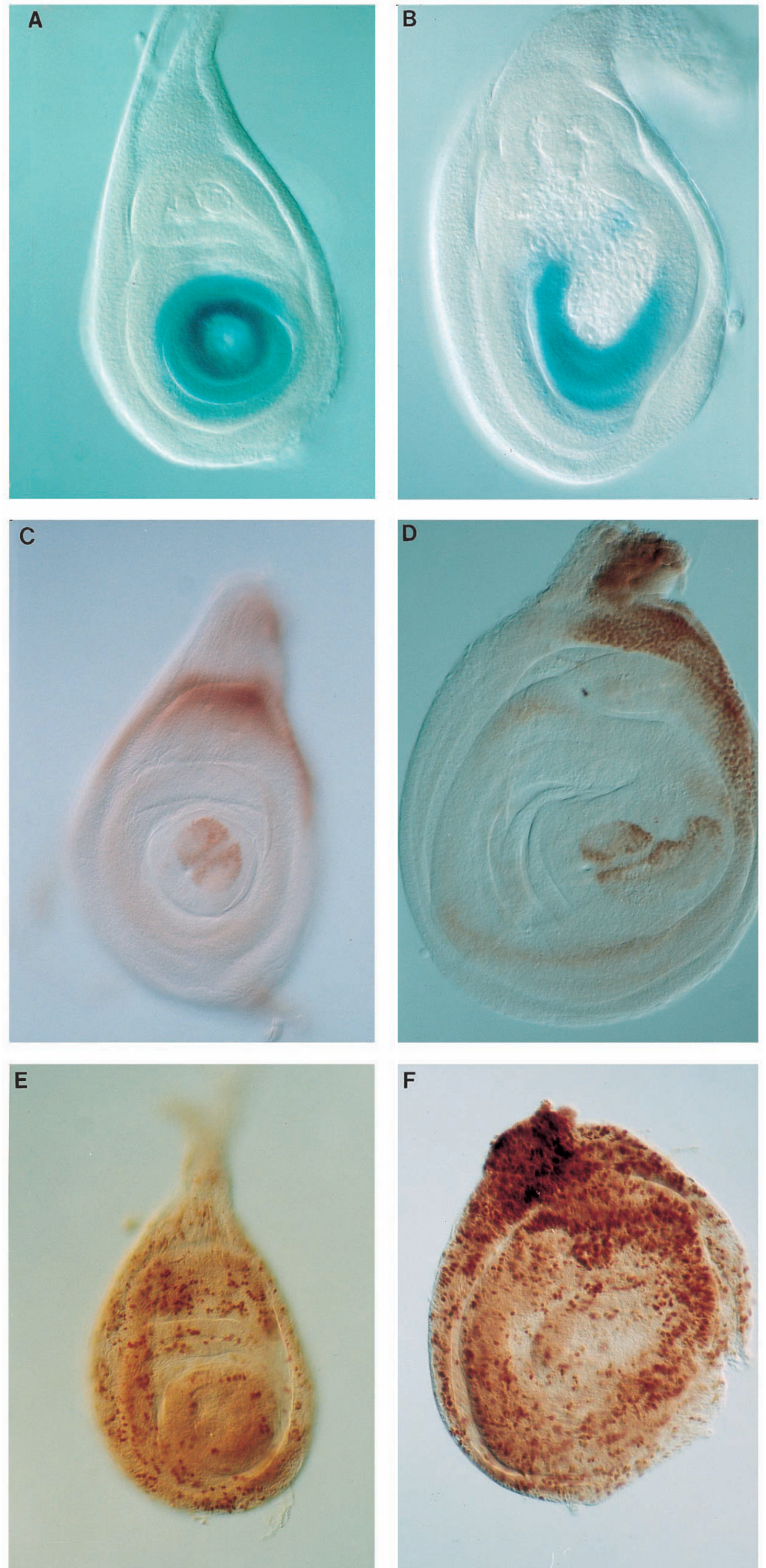


Fig. 5. Analysis of molecular markers in 1J3-UASwg^{ts} third instar leg discs. (A,B) *bab* expression in wild-type (A) or 1J3-UASwg^{ts} (B) discs. Expression of *bab* is monitored through the use of a *lacZ* insert at the *bab* locus (Godt et al., 1993). *lacZ* expression is detected by XGal staining (Ashburner, 1989). The circular pattern of wild-type discs is reduced to a semi-circle as a result of ectopic Wg. This 1J3-UASwg^{ts} disc has lost *bab* expression in most of the dorsal cells, but expression is variable in the dorsal anterior region. A similar effect is seen on *ap* expression (not shown). Dorsal cells in these discs are viable, as determined by their failure to absorb a vital dye (not shown). (C,D) Pattern of Al in wild-type (C) or 1J3-UASwg^{ts} (D) leg discs. Wild-type pattern of Al consists of 2 spots in the center of the disc (Campbell et al., 1993), which are expanded along the A/P border as a result of ectopic Wg. Ectopic expression of Wg in D results in distortion of the disc so that the A/P axis lies at an angle. (E,F) BrdU staining in wild-type (E) or 1J3-UASwg^{ts} (F) leg discs. Ectopic Wg in the 1J3 pattern results in increased incorporation in dorsal cells. This has been observed in 36 out of 55 discs scored (65%). A dorsal preference for BrdU incorporation in wild-type discs has been observed in 2/56 discs scored (3.6%). To determine whether this difference was due to a slight difference in developmental stage, this was repeated with discs from larvae that were precisely staged (see Materials and Methods) with similar results (not shown). A central quiescent region in E appears expanded in F. All discs developed at 16°C from first instar.

ectopic Wg expression (see Fig. 2C), suggesting that the effect is indirect.

wg function in proximodistal patterning

In addition to specifying information along the dorsoventral axis, *wg* has been implicated in the formation of the proximodistal axis (Struhl and Basler, 1993). Furthermore, it has been suggested to interact with *dpp* in this process through the specification of distal fate (Campbell et al., 1993). *dpp* is expressed along the A/P compartment border, with the level of expression being higher in the dorsal half of the disc (Masucci et al., 1990; Raftery et al., 1991). Thus Wg and high levels of Dpp normally intersect at a point in the center of the disc. To analyze the *wg/dpp* interaction, we superimposed expression of the two genes through a line in which GAL4 is controlled by *dpp* expression elements (Staehling-Hampton et al., 1994). Expression of Wg in the *dpp* domain causes the center of the disc to elongate (Fig. 6A). Expansion of the disc center is reflected in expanded Al expression (Fig. 6B). In addition, the pattern of BrdU incorporation reveals that a central region of quiescence is expanded (Fig. 6C). However, in a manner similar to the 1J3-UASwg^{ts} discs, a dorsal cap of cells is highly proliferative. Wg in the *dpp* domain also results in an incomplete circle of *bab* expression (not shown).

A likely explanation for the effects of Wg on proximal-distal organization and proliferation is that inappropriate specification of ventral fate in dorsal cells leads to pattern regulation, involving proliferation and intercalation, according to a model proposed by Meinhardt (1983). To determine whether these effects were a function of the ability of Wg to specify ventral fate, we examined H15 expression in *dppGAL4-UASwg*^{ts} discs (Fig. 6D). The A/P border lies beyond the region of the disc that can be ventralized in response to Wg, as is indicated by the normal pattern of H15 expression in these discs. Thus the effects of Wg on Al expression, proliferation, and dorsal differentiation are independent of its role in ventralization.

To determine the effects of this expression on P/D pattern, adult legs were examined. The most penetrant aspects of the phenotype are loss of dorsal structures and circumferential expansion (Fig. 7A). The presence of elements along the P/D axis indicate that, although the disc rings are grossly misshapen, the P/D axis forms and can evert during morphogenesis. In 3-5% of the legs, outgrowths from the proximodistal axis occur (Fig. 7B,C). These outgrowths are often completely ventralized. Surprisingly, the distal elements that arise from the expanded disc center, the claws, are variably present.

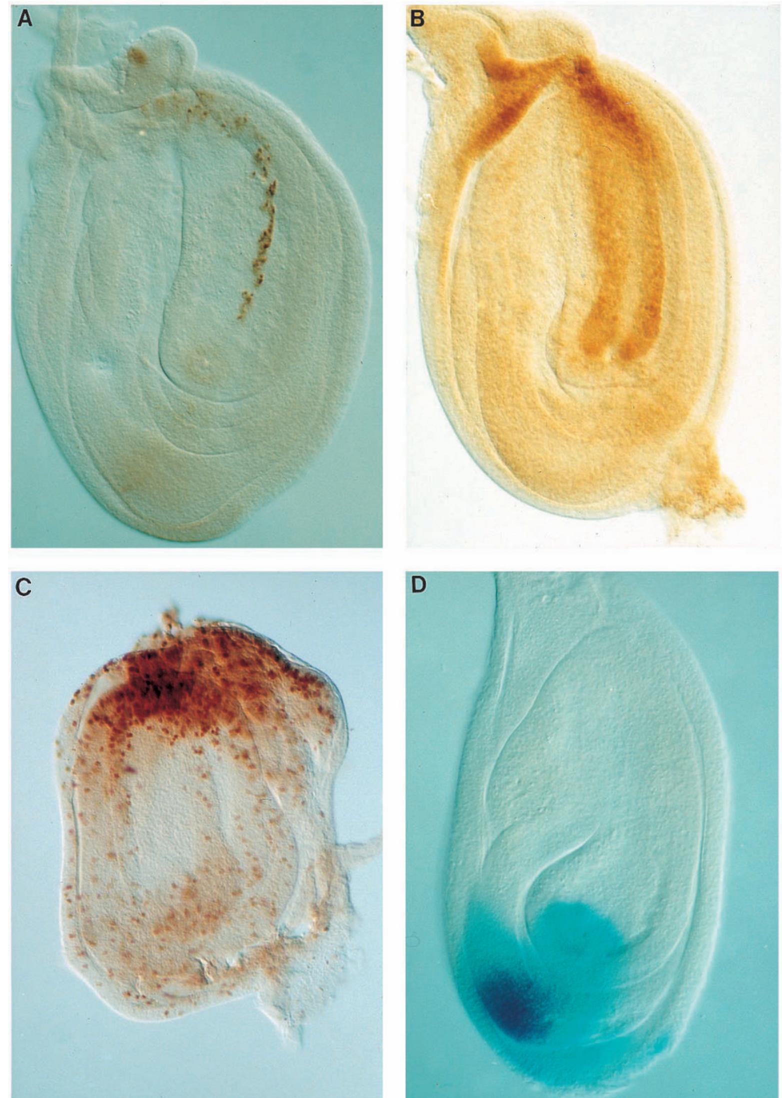


Fig. 6. The effects of *wg* expression at the A/P border. (A) Wg is detected in *dpp*GAL4-UASwg^{ts} discs along the dorsal A/P border in addition to its endogenous pattern. The center of the disc is expanded dorsally. (B) Expansion of the disc center is reflected in expansion of Al expression. The two central spots (see Fig. 4E) are converted to two stripes that abut the dorsal A/P border. (C) BrdU labeling reveals that the central quiescent region is also expanded, but in addition, a highly proliferative dorsal cap forms. Although the disc center is elongated making the P/D axis appear dorsally constricted, the overproliferation occurs throughout the dorsal P/D axis as is evident from staining in the concentric folds. The overproliferation has been observed in 42/61 discs scored (69%). (D) H15 expression in these discs is unchanged, revealing that the A/P border lies beyond the region that can be ventralized by Wg. All of these discs were exposed to ectopic Wg in the *dpp* domain beginning at 1st-2nd instar and maintained throughout development. Although the *dpp*GAL4 line shows variablility in expression at 16°C, discs grown under these conditions show distortion along the A/P border, and approximately 75% were similar to the discs shown here in the failure of the concentric rings to close dorsally.

DISCUSSION

Role of *wg* in establishing ventral fates

wg is expressed in the anterior ventral quadrant of the leg disc and is required for the specification of ventral fates (Baker, 1988; Couso et al., 1993). Struhl and Basler (1993) demonstrated that ectopic expression of low levels of *wg* in dorsal cells leads to partial ventralization and proposed that Wg specifies ventral fate in a concentration-dependent manner. In our experiments, we have shown that expression of high levels of ectopic *wg* and increasing Wg expression in the

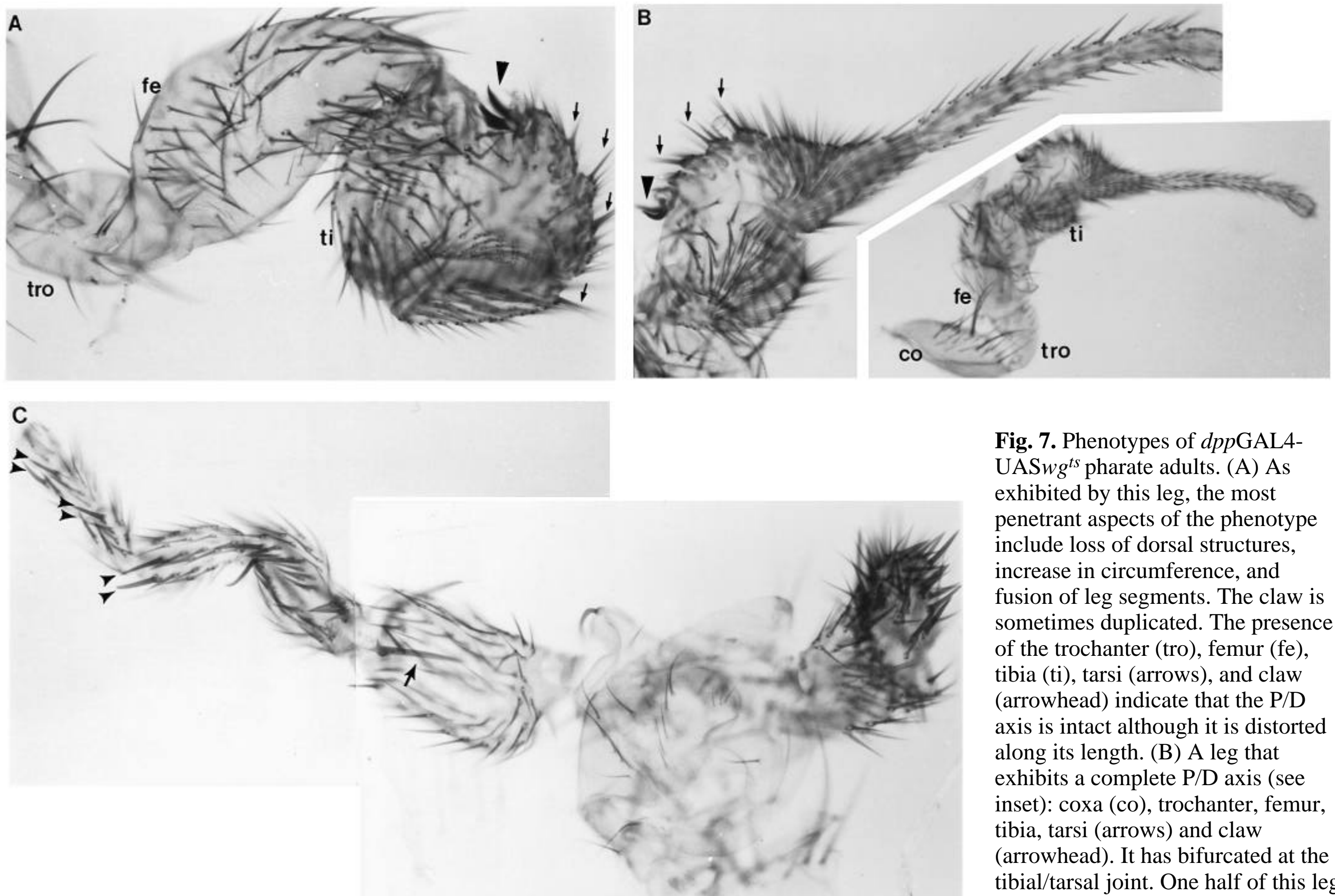


Fig. 7. Phenotypes of *dpp*GAL4-UASwg^{ts} pharate adults. (A) As exhibited by this leg, the most penetrant aspects of the phenotype include loss of dorsal structures, increase in circumference, and fusion of leg segments. The claw is sometimes duplicated. The presence of the trochanter (tro), femur (fe), tibia (ti), tarsi (arrows), and claw (arrowhead) indicate that the P/D axis is intact although it is distorted along its length. (B) A leg that exhibits a complete P/D axis (see inset): coxa (co), trochanter, femur, tibia, tarsi (arrows) and claw (arrowhead). It has bifurcated at the tibial/tarsal joint. One half of this leg exhibits ventral tarsal bristles (arrows) and a lack of dorsal structure so that the leg wraps around itself as in (A) to end in a claw (arrowhead). The other half of this leg maintains a tubular shape with ventral bristles but is missing a claw (see inset). (C) This proximally bifurcated leg exhibits an outgrowth consisting of a mass of ventral bristles (right) and a leg (left) that has ventral structures distal to the femur (tarsal bristles, arrowhead, and apical bristle, arrow) but is missing proximal elements and the claw. The ventral-most pattern elements exhibited by the left part of the leg are duplicated in a second focal plane (not shown). Flies were shifted to 16°C during second instar.

domain in which it normally acts are also associated with the expansion of ventral-lateral, rather than ventral fates (Fig. 3). Although we provide ectopic Wg in addition to endogenous Wg in ventral-lateral cells, we do not see expanded ventral-most structures nor is the region that expresses the marker H15 at high levels expanded. These data show that distorting the gradient of Wg protein in ventral cells does not change their fate. Therefore, Wg does not appear to function as a morphogen.

One possibility for the inability of 1J3-UASwg^{ts} to specify the complete set of ventral structures is that 1J3GAL4 may not direct ectopic expression early enough to induce complete ventralization. This is unlikely since loss of Zw3 activity late in second instar produces completely ventralized bristles (Fig. 4), indicating that dorsal cells can be ventralized during the time in which we know 1J3 to direct ectopic expression. A second caveat is raised by the use of a temperature sensitive protein: the mutant protein may not have wild-type activity at the permissive temperature. This is unlikely, given that wg^{ts} homozygous mutant individuals that are reared at the permissive temperature develop wild-type legs (Couso et al., 1994). Moreover, the caveat is eliminated through ectopic expression throughout the ventral region. Ectopic Wg is provided in addition to the endogenous protein, thereby altering the endogenous gradient such that high levels are present in all ventral cells. This situation does not result in an expansion of the most ventral surface of the leg.

That Wg does not specify fate in a graded manner is also suggested by Sampedro et al. (1993) and Noordermeer et al. (1994). These authors show that ubiquitous expression of Wg in the embryo can rescue the *wg* mutant phenotype to a significant degree. This indicates that a gradient of Wg may not be essential for embryonic epidermal patterning. In addition, although their data did not address the concentration dependence of Wg in ventral fate specification, Diaz-Benjumea and Cohen (1994) have suggested that Wg does not function as a morphogen in P/D axis organization (see below).

Ectopic expression of *wg* in the 1J3 domain has revealed a spatial restriction to its ventralizing capacity. The ventral marker H15 in this background is expanded into the anterior dorsal but not the posterior dorsal quadrant. Evidence that all cells have the capacity to be completely ventralized is provided by clones of *zw3* mutant cells. This demonstrates that signaling elements that restrict the ability of *wg* to ventralize the posterior dorsal quadrant sector are upstream of *zw3* and that

additional factors required for establishment of the most ventral fates are integrated through repression of *zw3*.

wg function in dorsal cells

Ectopic expression of *wg* in the dorsal part of the disc results in legs that are missing dorsal structures. In addition, expression of genes such as *bab* and *ap*, that define the commitment to differentiation of the tarsi, is missing in posterior dorsal cells and reduced in anterior dorsal cells (Fig. 5B). The tarsal segments are the most commonly missing elements and the most easily identifiable, but dorsal structures are also affected proximally (not shown). The loss of dorsal gene expression might be a result of one of three events: (1) cell death following ectopic *wg* expression, (2) transformation of tarsal cells to a different cell fate, i.e., a more proximal or distal fate, or (3) inhibition of dorsal cell determination. We have ruled out cell death since the failure of the dorsal cells in 1J3-UAS*wg^ts^* leg discs to absorb a vital dye indicates that they are viable. In addition, we found no evidence for a proximal transformation, although the possibility of a distal transformation was suggested by the duplication of claws. However, the duplication of claws is incompletely penetrant while dorsal structures are invariably missing from 1J3-UASwg^{ts} legs. Moreover, in *dpp*GAL4-UASwg^{ts} legs, dorsal structures are often lost in conjunction with loss of distal elements. Thus dorsal structures do not appear to be replaced by distal cuticular elements. Wg therefore appears to interfere with cell determination in dorsal cells.

A second effect of ectopic Wg in dorsal cells is overproliferation. This effect appears to be indirect since the region of intense BrdU labeling is largely outside the region of ectopic Wg expression (Figs 5F, 6C). One cause of overproliferation in discs is pattern regulation: cells that are inappropriately juxtaposed due to wounding undergo proliferation so that the missing cells are regenerated. Thus a possible reason for overproliferation in the presence of ectopic Wg might be that Wg supplies ventral information in the dorsal region and the surrounding cells proliferate to intercalate the intermediate cells. This, however, does not appear to be the reason for the overproliferation that we observe, since it occurs in the *dpp*GAL4-UASwg^{ts} discs, where Wg is unable to expand ventral fate in the majority of discs, as detected by H15 expression (Fig. 6C,D). The A/P border appears to be beyond the area that can respond to Wg by being ventralized, but proliferation is still induced in dorsal cells of these discs.

The overproliferation most often appears to result in a greater leg circumference but occasionally outgrowths occur (Fig. 7). Although ectopic H15 was not observed in the *dpp*GAL4-UASwg^{ts} discs, the ventral character of the outgrowths suggests that the A/P border lies just beyond the edge of the region that can be ventralized by Wg and that occasionally cells acquire ventral characteristics. As the cells proliferate, they may self-adhere and become extruded rather than being incorporated into the existing axis (see Garcia Bellido, 1966). The infrequency of this phenotype compared to the high frequency with which the over proliferation is observed indicates that the proliferative effect of Wg is independent of its ability to ventralize.

The biological relevance of Wg function in dorsal cells has to be questioned given the ventral restriction of *wg* expression in the wild-type disc. However, two situations exist in which dorsal cells receive Wg protein, suggesting that the effects that we observe are relevant to the normal biology of the disc. First, as discussed below, dorsal cells receive Wg at the center of the disc. Second, the induction of *wg* expression has been observed following wounding or cell death implicating it in the process of wound healing (W. Brook, A. Manoukian, S. Scanga, and M. Russell, unpublished observations). The imaginal discs form a blastema following wounding, with local dedifferentiation occurring in conjunction with proliferation (Abbott et al., 1981; Adler, 1981; Brook et al., 1993). Our data indicate that Wg might play a critical role in this process through its ability to interfere with dorsal differentiation. The fact that Wg can have this effect as late as third instar, when the dorsal-ventral pattern is already largely established (Schubiger, 1974), is consistent with the idea that Wg can induce a blastemal state.

Wg function in formation of the proximodistal axis

The role of *wg* in establishing the P/D axis is apparently quite complex, since both reduction of *wg* (Baker, 1988; Couso et al., 1993) and ectopic *wg* (Struhl and Basler, 1993; Campbell et al., 1993; Diaz-Benjumea and Cohen, 1994) can result in supernumerary limbs. Previous discussions of this subject have

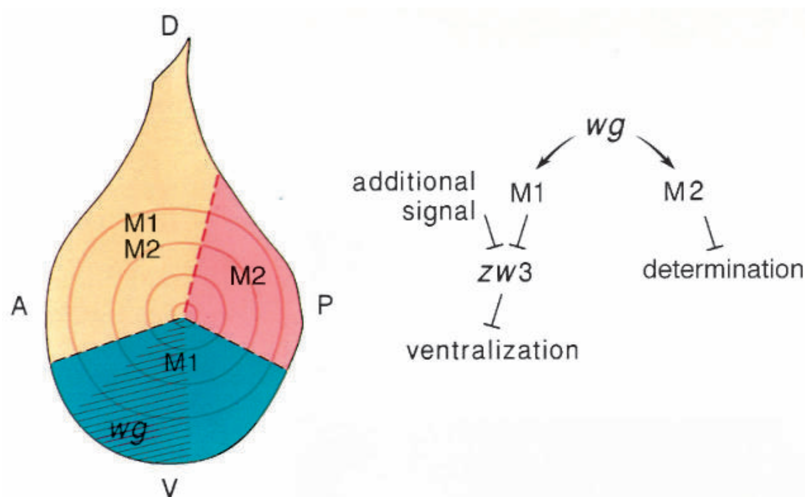


Fig. 8. Model for *wg* function in the leg disc. Endogenous *wg* is expressed in the anterior ventral quadrant (shaded area) and exerts a ventralizing influence throughout the ventral half of the disc (blue region) via the signaling mechanism, M1. Wg is sufficient to partially ventralize the anterior dorsal quadrant (yellow) as well, but is unable to specify ventral fate in the posterior dorsal quadrant (pink). This suggests that a component(s) of M1 is unavailable for interaction with Wg in the posterior dorsal quadrant. This component of M1 is apparently upstream of *zw3*, since loss of *zw3* bypasses this spatial restriction. Loss of Zw3 activity also results in complete ventralization, indicating that all factors necessary for this process are integrated through repression of *zw3*. In the dorsal compartment (yellow plus pink), Wg can interfere with cell fate determination. These effects of Wg are distinct from its function in ventralization and imply that a second mechanism for Wg signaling may exist (M2). Furthermore, the posterior dorsal cells can only respond to Wg via this pathway, so the effect on determination is more pronounced in this quadrant. The difference between M1 and M2 could conceivably entail either cooperation between Wg and different factors or interaction of Wg with different downstream effectors.

focused on the ventralizing function of *wg*. These discussions have led to variations of a model in which the P/D axis is proposed to form at the site of juxtaposition of 'sectors' of different positional values (French et al., 1976; Meinhardt, 1983; Gelbart, 1989; Couso et al., 1993; Bryant, 1993; Campbell et al., 1993; Diaz-Benjumea and Cohen, 1994). These values are thought to be specified by genes such as *wg, en, hh,* and *dpp*. According to this model, either loss of Wg or ectopic Wg could result in the inappropriate juxtaposition of cells with different positional information and could consequently lead to outgrowths of supernumerary axes through intercalation of intermediate positions. Our data indicate that this interpretation of Wg function is likely to be an oversimplification since they show that Wg can have effects other than the specification of ventral fate. The ectopic expression of Al and the distortion of the concentric rings of the discs without concomitant ventralization by Wg suggests that the influence of Wg on the P/D axis reflects this second function of Wg rather than its function in the specification of ventral fate.

Conclusions

Our results suggest a model for Wg function in the leg disc in which Wg achieves separate functions via spatially regulated mechanisms (M1 and M2; see Fig. 8). Although *wg* has been known to function in axial patterning of the imaginal discs, the way in which it acts has been unclear. Our data indicate that Wg acts cooperatively rather than as a concentration-dependent morphogen in the specification of ventral fate. In addition, we show that it can inhibit dorsal commitment to a determined state via a spatially distinct mechanism. Further analysis of Wg function in axial pattern will require consideration of both of these effects. Likewise, understanding Wg signaling will require consideration of the possibility of multiple signaling mechanisms.

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