wingless acts through the *shaggy/zeste-white 3* kinase to direct dorsal-ventral axis formation in the *Drosophila* leg

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SUMMARY

The secreted glycoproteins encoded by *Wnt* genes are thought to function as intercellular signaling molecules which convey positional information. Localized expression of Wingless protein is required to specify the fate of ventral cells in the developing *Drosophila* leg. We report here that Wingless acts through inactivation of the *shaggy/zeste white 3* protein kinase to specify ventral cell fate in the leg. Ectopic expression of Wingless outside its normal ventral domain has been shown reorganize the dorsal-ventral axis of the leg in a non-autonomous manner. Using genetic mosaics, we show that cells that lack *shaggy/zeste white 3* activity can influence the fate of neighboring cells to reorganize dorsal-ventral pattern in the leg, in the same manner as Wingless-expressing cells. Therefore, clones of cells that lack *shaggy/zeste white 3* activity exhibit all of the organizer activity previously attributed to Wingless-expressing cells, but do so without expressing *wingless*. We also show that the organizing activity of ventral cells depends upon the location of the clone along the dorsal-ventral axis. These findings suggest that Wingless protein does not function as a morphogen in the dorsal-ventral axis of the leg.

Key words: *zeste-white 3, shaggy, wingless*, organizer, cell interaction, pattern formation, limb development

INTRODUCTION

Grafting experiments performed on the limb buds of vertebrate embryos and cockroaches, the imaginal discs of *Drosophila*, and the body segments of a variety of insects have suggested that cells acquire information about their prospective fate with reference to their position (reviewed in Wolpert, 1969; Lawrence, 1972; French et al., 1976). This view has received substantial support from the genetic and molecular analysis of segmentation in *Drosophila*. Many of the genes that are thought to generate positional information along the anterior-posterior axis of the embryonic segment encode molecules implicated in intercellular signaling and signal transduction (reviewed in Ingham and Martinez Arias, 1992). Thus, pattern specification can be considered as a problem of intercellular signaling. The group of cells that constitute the primordium of an appendage, or of an embryonic segment, can be thought of as a developmental field which is patterned with reference to a common set of positional signals (Wolpert, 1969). A subset of cells in the field must serve as the source of positional information. These cells behave as an organizer region which influences the fate of other cells in the field. To understand how spatial pattern is generated, we must identify the genes that encode the pattern-organizing substances and understand both the cellular basis for the propagation of positional information and the molecular basis by which cells interpret this information to adopt specific fates (reviewed in Ingham and Martinez Arias, 1992; Jessell and Melton, 1992; Greenwald and Rubin, 1992).

The *wingless* gene (*wg*) and its vertebrate homologues, the *Wnt* genes, have been the focus of particular interest as candidates to encode positional information (reviewed in Nusse and Varmus, 1992). The *wg* and *Wnt-1* genes encode secreted glycoproteins, which are thought to function as intercellular signaling molecules (Papkoff, 1989; van den Heuvel et al., 1989; Papkoff and Schryver, 1990; Bradley and Brown, 1990; Gonzalez et al., 1991). Localized expression of the Wg protein in a subset of cells in the embryonic segment of *Drosophila* has been shown to affect the developmental fate of neighboring cells (DiNardo et al., 1988; Martinez Arias et al., 1988; Heemskerk et al., 1991; Bejsovec and Martinez Arias, 1991; Dougan and DiNardo, 1992). The secreted Wg protein can be detected in multivesicular bodies in neighboring cells, suggesting that Wg functions as a paracrine signal (van den Heuvel et al., 1989). Paracrine signaling by Wnt proteins has been reproduced in vitro using co-culture assays, in which a population of Wg- or Wnt-1-expressing cells can be shown to affect the differentiation of a second cell type in a non-autonomous manner (Jue et al., 1992; Cumberledge and Krasnow, 1993; Parkin et al., 1993). *wg* is expressed in a spatially restricted pattern in the embryonic segments of *Drosophila* (Baker, 1987; Rijsewijk et al., 1987). Mutations that remove *wg* function lead to changes in cell fate throughout the segment, suggesting that Wg might contribute to the proper specification of the fates of both adjacent and more distant cells (Baker, 1988b; Martinez Arias et al., 1988; van den Heuvel et al., 1989; Peifer et al., 1991; Gonzalez et al., 1991). Moreover, ectopic expression of *Wnt* genes in spatially

inappropriate patterns leads to respecification of cell fate in *Drosophila* and *Xenopus* embryos (McMahon and Moon, 1989; Sokol et al., 1991; Smith and Harland, 1991; Noordermeer et al., 1992; but see Sampedro et al., 1993).

Recently, Struhl and Basler (1993) have proposed that the secreted Wg protein functions as a concentration-dependent morphogen to specify cell fate along the dorsal-ventral axis of the adult leg of *Drosophila*. *wg* transcript is expressed in a ventral-anterior sector of the developing leg imaginal disc (Baker, 1988a; Peifer et al., 1991; Couso et al., 1993). Mutations that reduce *wg* gene activity lead to loss of ventral structures from the leg disc, and to foreshortening of the leg along the proximal-distal axis (Baker, 1988a; Peifer et al., 1991; Couso et al., 1993). By producing clones of cells that constitutively express *wg*, Struhl and Basler (1993) have shown that ectopic expression of Wg protein in dorsal cells is sufficient to reprogram their fate from dorsal to ventrolateral. Furthermore, the presence of a clone of ventrolateral cells in an ectopic dorsal location reprograms the fates of surrounding cells to adopt more ventral identities. These experiments suggested that the cells expressing *wg* can serve as a ventral organizer and that the secreted Wg protein may directly specify the fate of nearby cells.

To investigate the mechanism by which *wg* organizes dorsal-ventral pattern, we have compared the effects of producing clones of cells in the leg imaginal disc which secrete Wg protein with the effects of producing clones of cells mutant for *shaggy/zeste white 3* (*sgg/zw3*). The *sgg/zw3* gene encodes a predicted serine-threonine protein kinase (Bourouis et al., 1990; Siegfried et al., 1990) which acts in the signal transduction pathway that mediates the *wg* signal in the embryonic segment (Siegfried et al., 1992). Binding of Wg protein to its receptor is thought to lead to inactivation of the otherwise constitutively active *sgg/zw3* kinase (Siegfried et al., 1992). Consequently, eliminating the gene encoding the *sgg/zw3* kinase from a clone of cells is expected to be equivalent to providing those cells with the Wg signal. We report here that clones of *sgg/zw3* mutant cells exhibit all of the properties attributed to the ventral organizer. Cells that lack *sgg/zw3* activity can influence the fate of surrounding cells and reorganize dorsal-ventral pattern in the developing leg. Furthermore, we show that organizer activity depends on interaction between ventral cells and dorsal cells near the anterior-posterior compartment boundary. These results suggest that organizer activity is generated by interaction between populations of distinctly specified cells.

MATERIALS AND METHODS

Production of somatic mosaic clones

The null allele *sgg/zw3*[D127] was used for making clones of cells lacking *sgg/zw3* function. The *sgg/zw3*[D127] chromosome was marked with the cell-autonomous recessive bristle marker *forked*[36a] by meiotic recombination. *sgg/zw3* is located at meiotic map position 1-1.3 and *forked* is located at 1-56.7 on the X chromosome. To permit recovery of large clones of *sgg/zw3* mutant cells, the mutant cells were given a relative growth advantage introducing a strong *Minute* mutation, M(1)o, on the *sgg/zw3*[+] chromosome (Ferrus, 1975). The *sgg/zw3 forked* mutant chromosome was introduced through the females. The *Minute* mutant chromosome was introduced through males carrying a *Minute*[+] duplication on chromosome 3 (Tp[1;3]

f[+]71b). Embryos from this cross were collected for 24 hour periods, aged at 25°C and irradiated at 60 or 84 hours (±12 hours) after egg laying (corresponding to 1st and 2nd larval instars, taking into account the developmental delay caused by the *Minute* mutant). Irradiation was performed at 150 kV, 5 mA for a total does of 1000 Rad using a Torrex 120/150D X-ray source. X-ray-induced somatic recombination proximal to *forked* results in clones of cells that are homozygous mutant for *sgg/zw3* and *forked* and which are M(1)o[+] (illustrated in Fig. 2). Clones of cells expressing *wg* were produced by crossing strains carrying the *hsp70-flp* and *Act5C>y[+]>wg* transgenes and treating doubly heterozygous larvae to a heat shock during the early second larval instar, as described by Struhl and Basler (1993).

Antibody labeling of imaginal discs

Cells expressing *wg* were visualized by labeling imaginal discs from larvae carrying a P-element insertion at the *wg* locus, *wg*[rO727], with antibody to β-galactosidase. Histochemical labeling were carried out as described previously (Diaz-Benjumea and Cohen, 1993).

Fly strains

The molecular characterization of *sgg/zw3*[D127] as a null allele is described in Ruel et al. (1993a). Strains carrying the *hsp70-flp* and *Act5C>y[+]>wg* transgenes used to make *wg*-expressing clones were provided by Konrad Basler (Struhl and Basler, 1993). The nuclear Wg-lacZ reporter line, *wg*[rO727] was provided by Ulrike Gaul. All other strains are described in Lindsley and Zimm (1992).

RESULTS

The dorsal-ventral patterning system of the leg primordia is inherited from the embryonic segment

The adult appendages of *Drosophila* originate as small clusters of cells in the embryonic ectoderm, which invaginate midway through embryogenesis to form simple epithelial sacs known as imaginal discs (Auerbach, 1936; Madhavan and Schneiderman, 1977; Bate and Martinez Arias, 1991; Cohen et al., 1991). During early postembryonic growth, the epithelium on one side of the sac becomes columnar, while the other remains as a thin squamous layer. This morphologically simple arrangement is maintained until the third larval instar, when continued growth throws the columnar epithelium into a series of folds which project into the lumen of the sac. Fate mapping studies have shown that the central-most folds of the columnar disc epithelium form the distal-most tarsal segments of the adult leg, while progressively more peripheral folds form more proximal leg segments (Schubiger, 1968, illustrated in Fig. 1).

Specification of the thoracic imaginal primordia in the embryo requires the activity of the segment polarity gene *wg* (Simcox et al., 1989). Localized expression of Wg in the embryonic segment provides a positional signal which allocates cells to the disc primordia (Cohen, 1990; Cohen et al., 1993). When the thoracic imaginal primordia are allocated at ~4.5 hours of embryogenesis, the row of cells expressing *wg* is incomplete along the dorsal-ventral axis of the embryonic segment. Consequently, *wg* is expressed in cells in the ventral but not in the dorsal portion of the nascent disc primordium (Fig. 1A,C). During postembryonic growth of the leg disc, *wg* expression is maintained in cells that occupy a corresponding ventral position, with the result that the domain of *wg* expression forms a wedge-shaped sector of the disc (Fig. 1B,D, see also Couso et al., 1993). Eversion of the disc during mor-
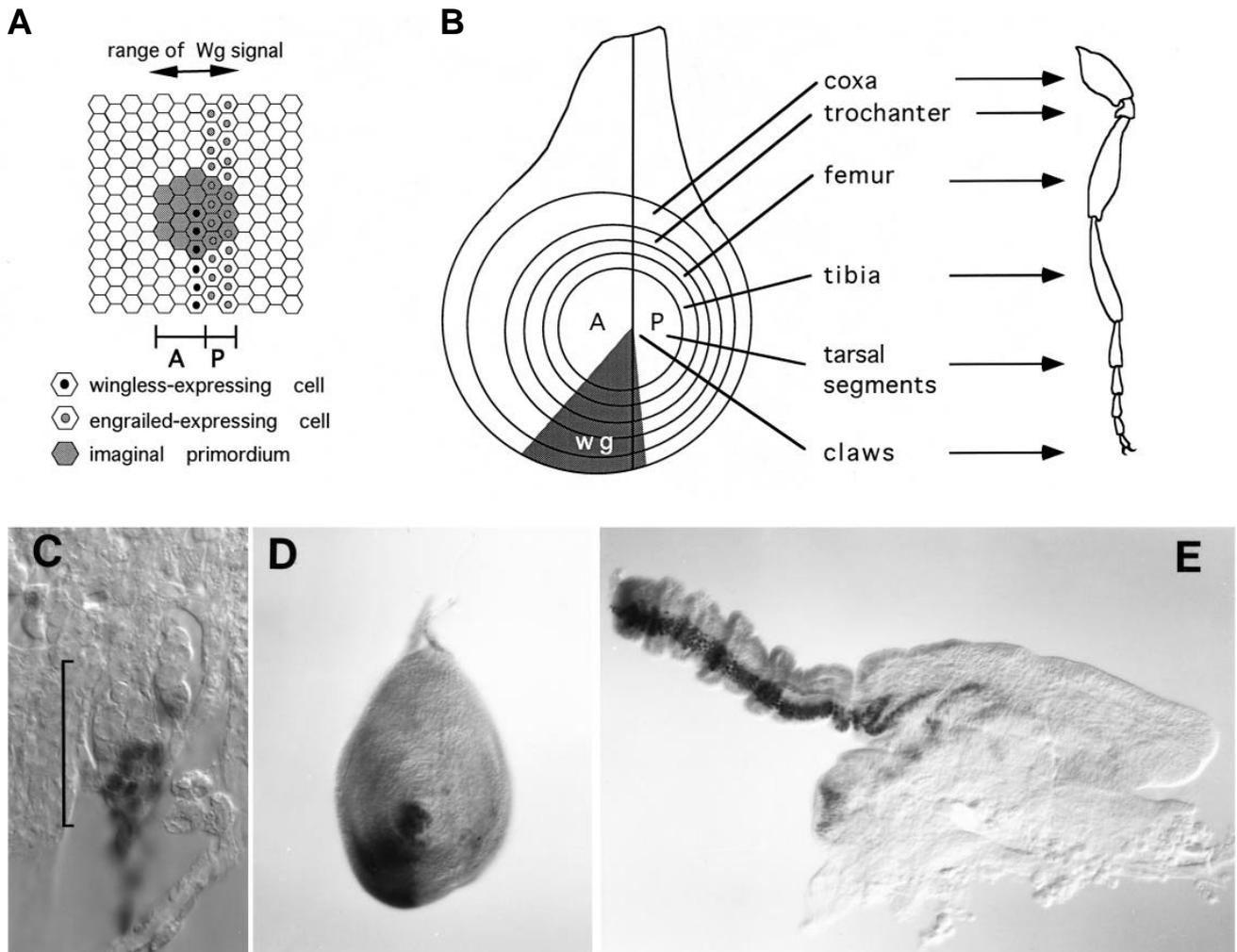
Fig. 1. Spatial organization of the leg imaginal disc revealed by *wg* expression. (A) Schematic representation of the spatial relationship between cells expressing *wg* and the imaginal disc primordium in the embryo. The secreted Wg protein provides a positional signal to recruit nearby cells into the disc primordium. The row of *wg*-expressing cells is incomplete along the dorsal-ventral axis of the embryonic segment at the stage when the disc primordium is formed. Consequently, the domain of *wg* expression lies in the ventral region of the primordium (compare with C). Cells in the embryonic ectoderm are depicted as hexagons. The cells of the imaginal primordium are filled in gray. Cells expressing *wg* are indicated by black circles. The posteriorly adjacent *engrailed*-expressing cells are indicated by grey circles. A and P indicate anterior and posterior compartments of the nascent primordium. The drawing is oriented with anterior to the left and ventral down (see also Cohen and DiNardo, 1993; Couso and Gonzalez-Gaitan, 1993). (B) Schematic representation of the relationship between the mature leg imaginal disc from a third instar larva and the adult leg. The disc is a single-layered epithelial sac, which forms a columnar epithelium on one side and a thin squamous epithelium on the other. In this diagram, the disc is represented as a flat sheet, with the columnar epithelium in the center and the squamous epithelium at the periphery (following Schubiger, 1968). During growth, the columnar epithelium is thrown into a series of folds, represented here as concentric circles. The central fold corresponds to the primordia of the five tarsal segments. The next fold corresponds to the tibia, and progressively more peripheral folds correspond to more proximal leg segments. The outer squamous epithelium is known as the peripodial membrane and forms the ventral thoracic body wall (see e.g. Milner et al., 1984). A denotes the anterior compartment. P denotes the posterior compartment. The domain of *wg* expression is indicated as a shaded wedge (compare with panel D). (C-E) Leg imaginal discs labeled to visualize the pattern of *wg*-expression. (C) *wg* expression in an invaginated leg disc from a late embryo. The disc forms a discrete cluster of cells, indicated by the bracket. *wg*-expressing cells are located in the ventral-most region of the disc. *wg*-expressing cells are visualized by expression of a nuclear β-galactosidase protein encoded by a P-element enhancer detector insertion in the *wg* gene. (D) *wg* expression forms a wedge-shaped sector in the mature third instar leg disc. The wedge pattern reflects the domain of *wg* expression throughout larval development of the leg disc. (E) The wedge-shaped sector of *wg* expression can be seen to form an elongated stripe of cells during eversion of the leg. The stripe is about 5 cells wide and is located in the ventral surface of the leg. The stripe runs from the distal tip of the tarsus to the most proximal segment of the leg.

phogenesis reveals that this sector corresponds to a stripe of ventral cells that runs along the entire proximal-distal axis of the leg (Fig. 1E). Mutations that reduce *wg* gene activity lead to loss of ventral structures in the leg and to symmetric duplications of normal dorsal structures, suggesting that localized expression of *wingless* is required to specify the fate of cells

Fig. 2. Scheme for producing clones of genetically marked cells lacking activity of the *sgg/zw3* gene. Clones of genetically marked cells, mutant for the *sgg/zw3* gene were generated by X-ray-induced somatic recombination. The *sgg/zw3* mutant chromosome was marked with the cell-autonomous, recessive bristle marker *forked*. The *forked* mutation alters the morphology of bristles and therefore provides an innocuous genetic marker that allows the *sgg/zw3* mutant cells to be identified in the adult leg. X-ray-induced recombination proximal to *forked* leads to production of unequal daughter cells at the next cell division. One daughter cell is homozygous mutant for both sgg^{D127} and *forked*, and is *Minute⁺*. The sister chromosome carries a *Minute* mutation, which slows the rate of cell division in a dominant, cell-autonomous manner (Morata and Ripoll, 1975). Therefore the *sgg/zw3*, *forked* mutant cells have a growth advantage over the surrounding heterozygous cells, which carry the Minute mutation, allowing the production of relatively large clones of mutant cells.

recombination event proximal to *sgg/zw3*

homozygous mutant for sgg^{D127} & *f*

homozygous *Minute*; dead
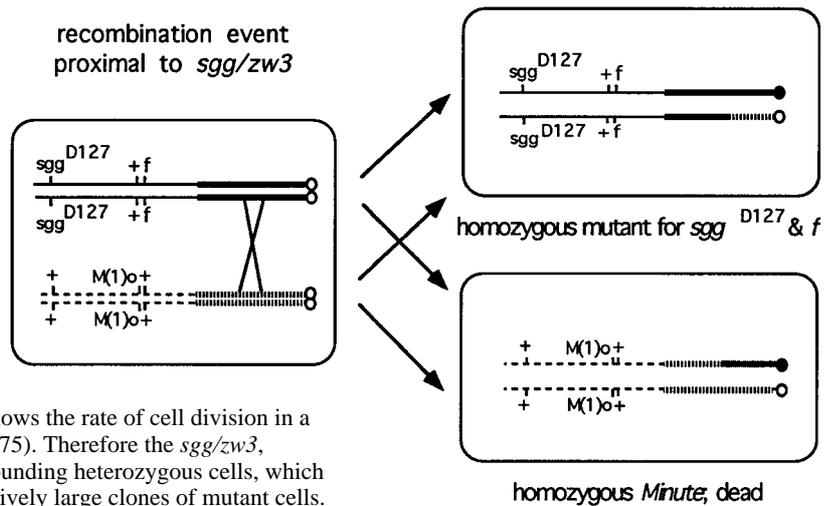
in the ventral leg (Baker, 1988a; Peifer et al., 1991; Couso et al., 1993). These observations suggest that the imaginal primordia may simply inherit the localized domain of *wg* expression from the embryo. Since the *wg*-expressing sector occupies a ventral region of the disc, the positional information provided by localized expression of *wg* may be used in dorsal-ventral patterning of the discs rather than in anterior-posterior patterning as in the embryonic segment (see legend to Fig. 1 for details).

Removal of *sgg/zw3* activity is sufficient to specify ventral cell fate in the leg

In the embryo, Wg signaling is thought to be mediated by inactivation of the serine/threonine protein kinase encoded by the *shaggy/zeste white 3* gene (*sgg/zw3*; Siegfried et al., 1992). We sought to mimic the effects of providing the Wg signal in the developing leg imaginal discs by producing clones of cells that are mutant for the *sgg/zw3* gene. We have made clones of cells that are homozygous mutant for the D127 allele of *sgg/zw3* by X-ray-induced mitotic recombination (illustrated in Fig. 2). $sgg/zw3^{D127}$ has been shown to lack all protein products encoded by the gene, and therefore can be considered to be a complete lack-of-function allele (Ruel et al., 1993a). Consequently, if all of the functions of Wg signaling are mediated through *sgg/zw3*, mutant cells in the clone should develop as though they had received the maximum possible dose of the Wg signal.

Clones of cells mutant for $sgg/zw3^{D127}$ differentiate as the

Fig. 3. Comparison of phenotypes produced by *sgg/zw3* mutant clones and by *wg*-expressing clones. (A,B) *sgg/zw3* mutant clones in the leg; (C,D) clones of cells expressing *wg*. (A) A *sgg/zw3* mutant clone in the ventral region of the leg. The mutant cells can be recognized by the production of *forked* mutant bristles. The location of the clone in the first tarsal segment is indicated by thin arrows. The mutant clone develops normal ventral structures and does not perturb the organization of the leg. The diagram to the right shows a schematic representation of the ventrally located *sgg/zw3* mutant clone shown in A. Localized expression of *wg* specifies the fate of ventral cells in the disc (darkly shaded sector). Clones of cells mutant for *sgg/zw3* which lie in the *wg*-expressing domain develop as normal ventral cells. In the example presented here, the clone lies in the anterior compartment and differentiates ventral bristles typical of row 4. When such a clone is in the posterior compartment the mutant cells differentiate normal row 5 bristles (not shown). Note the local increase in the density of bristles within the *sgg/zw3* mutant clone. The extra bristles are always of ventral type, and are typically found in well-organized rows (depicted as extra row 4 bristles in the diagram). *sgg/zw3* has been implicated in the process of lateral inhibition, in which the ectodermal cell fated to become a bristle-forming neuroblast inhibits neighboring ectodermal cells from becoming neuroblasts (see Heitzler and Simpson, 1991; Ruel et al., 1993b). The increase in bristle density that we observe in the *sgg/zw3* mutant clones apparently reflects a failure of lateral inhibition. Note, for example, the apical bristle, a ventral marker at the distal end of the tibia of the second leg, is included in the mutant clone and is duplicated (arrowhead). (B) A *sgg/zw3* mutant clone in the dorsal region of a third leg. The clone is visible in the transverse

rows of the tibia, proximal to the branch point of the bifurcation in the first tarsal segment, and extends through the tarsal segments (arrows). The *sgg/zw3* mutant cells differentiate as the ventral row 4 cells in the duplicated leg. The presence of a dorsally located mutant clone causes a bifurcation of the leg. Note that the polarity of the supernumerary leg is opposite to that of the endogenous leg. The leg bifurcates dorsally, and the ventral structures are located on opposite sides (the claws of the supernumerary leg point away from those of the endogenous leg). The diagram to the right shows a schematic representation of the relationship between the mutant clone and the supernumerary leg. Note that the duplicated leg makes only anterior (row 4) ventral bristles, indicating that the clone is in the anterior compartment. To cause the bifurcation, the mutant cells must respecify the fates of surrounding wild-type dorsal cells toward more ventral identities. Bifurcations of this type can be found in all three legs as well as in the antenna and maxillary palp, which are ventral appendages homologous to the leg (see e.g. Cohen and Jürgens, 1989). (C) A ventrally located *wg*-expressing clone in the femur develops normally. The clone is marked by *yellow* bristles. The arrows indicate the position of the clone in the femur. The clone extends distally into the tibia. (D) Dorsally located *wg*-expressing clone causes a bifurcation of the leg in the first tarsal segment. The duplicated leg has opposite polarity to the endogenous leg. The *wg*-expressing cells adopt ventrolateral fates. Therefore the duplicated leg is circumferentially incomplete, and lacks the most ventral cell types. The phenotypes caused by clones of *wg*-expressing cells have been describe by Struhl and Basler (1993), and are shown here only for comparison with the effects of producing *sgg/zw3* mutant clones.

ventral-most cell types in the leg independent of the position of the clone with respect to the dorsal-ventral axis of the leg (*n*=185 clones; Figs 3, 5). These results indicate that removing *sgg/zw3*[D127] function is sufficient to specify the fate of the mutant cells as ventral-most, suggesting that *wg* acts through *sgg/zw3* to specify ventral cell fate in the leg. For comparison, we have confirmed the observations of Struhl and Basler (1993) that *wg*-expressing clones of cells that lie outside of the endogenous ventral region of the leg adopt ventrolateral cell fate (*n*=112 clones; Fig. 3D). Struhl and Basler reported that these clones express Wg at a low level compared to that observed in the endogenous ventral region of the leg, leading to specification of ventrolateral fate. Our finding that *sgg/zw3* mutant cells adopt ventral-most identity is consistent with their proposal that high levels of Wg activity are required to specify the most ventral fates.

Clones of cells lacking *sgg/zw3* can reorganize the dorsal-ventral axis of the leg

Clones of cells mutant for *sgg/zw3* in the ventral region of the leg differentiate as normal ventral cells and do not perturb the global organization of the leg (*n*=87 clones located ventrally; Fig. 3A). Thus removing the activity of the *sgg/zw3* from the region of the leg where *wg* is normally expressed has no effect on the organization of dorsal-ventral pattern in the leg. Similarly, clones of cells that constitutively express *wg* within the ventral region of the leg differentiate as ventral cells and do not perturb the organization of the leg (Fig. 3C, Struhl and Basler, 1993). The only discernible phenotype associated with ventrally located *sgg/zw3* mutant clones is a local increase in bristle density due to a failure in lateral inhibition of neuronal fate specification (see Heitzler and Simpson, 1991; Ruel et al., 1993b; and legend to Fig. 3 for details).

By contrast, *sgg* mutant clones that lie in the dorsal region of the leg can cause a global reorganization of dorsal-ventral pattern, which leads to bifurcation of the leg. These legs branch from the dorsal surface of the endogenous leg and the *sgg/zw3* mutant clone runs along the ventral side of the supernumerary leg (Fig. 3B). The *sgg/zw3* mutant cells differentiate exclusively as ventral-most cell types, while the remainder of the duplicated leg derives from wild-type cells (see illustration, Fig. 3). Thus the *sgg/zw3* mutant cells have the capacity to respecify the developmental fates of normal dorsal cells so as to reorganize a circumferentially complete supernumerary dorsal-ventral leg axis. Clones of cells that constitutively express *wg* in dorsal regions of the leg cause a similar bifurcation phenotype, except that the duplicated leg lacks the most ventral cell types (Fig. 3D; Struhl and Basler, 1993). These data indicate that clones of cells that lack *sgg/zw3* activity exhibit all of the properties of a ventral pattern organizer. These findings raise the possibility that the only requirement for *wg* in organizing the dorsal-ventral axis of the leg may lie in locally inactivating *sgg/zw3*.

Organizing activity is a property of ventral cells

Although *sgg/zw3* is thought to function in the signal transduction pathway downstream from the *wg* signal (Siegfried et al., 1992), we considered the possibility that the *sgg/zw3* mutant cells might express *wg* for some unanticipated reason, and thus exhibit organizer activity. If this were the case we would expect the properties of *wg*-expressing clones and *sgg/zw3* mutant clones to be comparable. However, we observe a difference between the ability of *wg*-expressing clones and *sgg/zw3* mutant clones to produce bifurcations in which the duplicated leg distalizes significantly. *wg*-expressing clones which originate in the tibia or femur can cause complete distalization of the duplicated leg, including formation of tarsal segments with claw organs (Fig. 4A,B; Table 1). Bifurcations produced by *sgg/zw3* mutant clones at comparable positions in the tibia or femur are unable to extend beyond the end of the segment in which the bifurcation occurred (Fig. 4C,D; Table 1). Although the dorsal-ventral organizing activity of *sgg/zw3* mutant and *wg*-expressing clones are comparable in the distal region of the leg (e.g. Fig. 3), there is a striking difference capacity of these clones to direct distalization in the proximal leg.

As an additional control, we examined *wg* expression in imaginal discs from larvae in which clones of *sgg/zw3* mutant cells were produced. We made use of the *wg*-lacZ P-element insertion to visualize *wg* expression, since detection of expression of the enhancer detector is more sensitive than detection of the endogenous Wg protein. We saw no evidence of ectopic *wg* expression in over two thousand leg and antenna discs, although we did find several bifurcations of the type generated by *sgg-zw3* mutant clones (data not shown). Based on these two sets of data, we conclude that the organizing activity

of *sgg/zw3* mutant cells cannot be a consequence of mis-expressing *wg*. Rather, it appears that organizing activity is a property of ventral cells, independent of whether these cells secrete Wg protein.
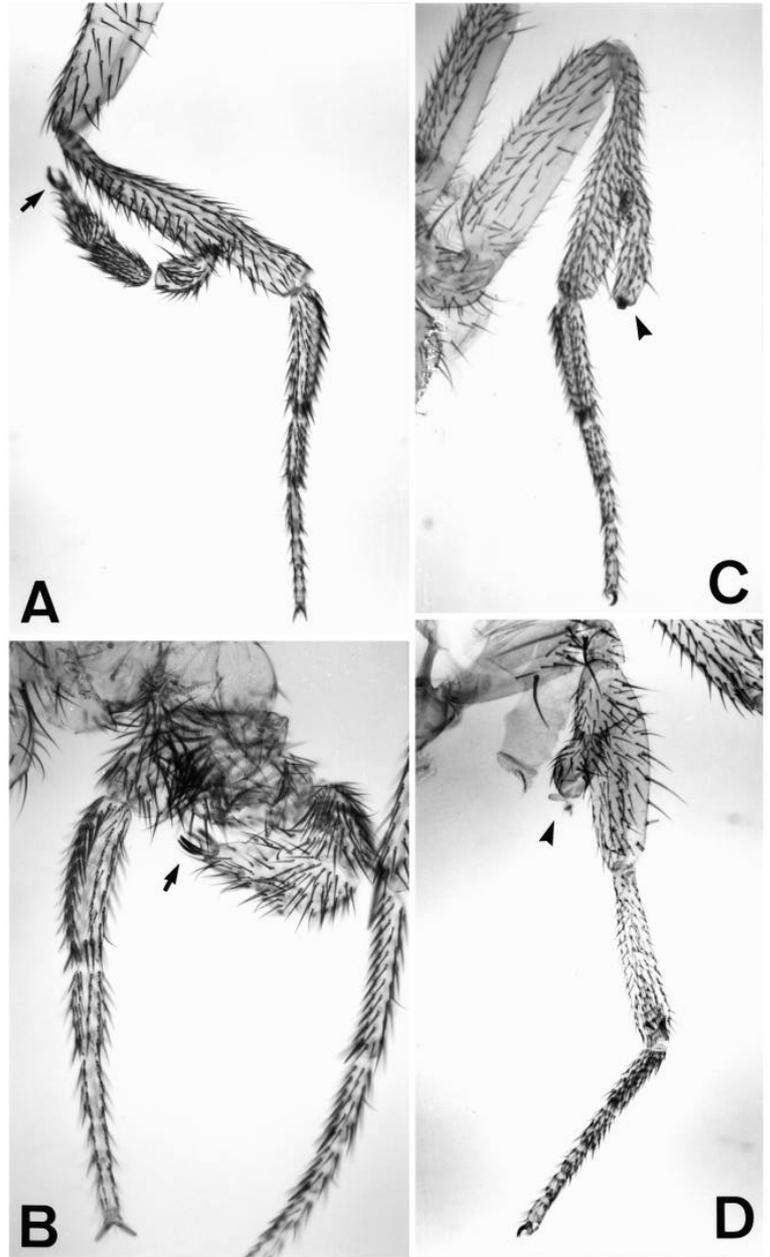

Fig. 4. *sgg/zw3* mutant clones and *wg*-expressing clones produce different phenotypes in proximal leg segments. (A,B) Distally complete supernumerary legs produced by clones of *wg*-expressing cells. (A) The bifurcation occurs on the dorsal side of the tibia. (B) The bifurcation occurs in the femur (not visible in this plane of focus). The duplicated leg is somewhat twisted. Arrows is A and B indicate claw organs produced at the distal tip of the duplicated legs. (C,D) Distally incomplete supernumerary legs associated with *sgg/zw3* mutant clones. (C) The bifurcation occurs on the dorsal side of the tibia. The duplicated leg extends distally to the end of the tibia, but does not make more distal leg structures (arrowhead). Compare with A. (D) The bifurcation occurs on the dorsal side of the femur, but does not distalize. See Table 1 for a comparison of a large number of bifurcations produced using the two systems.

Table 1. Distalization of duplicated legs produced by *wg*-expressing clones or *sgg/zw3* mutant clones

	wg-expressing clones			*sgg/zw3* mutant clones		
Leg segment	No. of bifurcations	No. of distalized	%	No. of bifurcations	No. of distalized	%
coxa	1	0	0	3	0	0
trochanter	3	2	67	6	0	0
femur	15	8	53	10	0	0
tibia (proximal)	16	6	38	7	0	0
sum proximal	35	16	**46%**	26	0	**0%**
tibia (distal)	17	11	65	8	6	75
tarsus (1-5)	26	18	69	17	8	47
sum distal	43	29	**67%**	25	14	**56%**

Supernumerary legs caused by *wg*-expressing clones and by *sgg/zw3* mutant clones differ significantly in their ability to produce distal leg structures, depending on the location of the bifurcation along the proximal distal axis of the leg. The number of duplicated legs is presented according to the leg segment in which the leg bifurcated. A supernumerary leg is considered to have distalized if it produced structures from the next segment distal to its origin (e.g. a bifurcation in the femur had to produce tibia or more distal structures to be considered distalized). The ability of duplicated legs to distalized was found to correlate with the segment in which the bifurcation of the leg originates. None of the proximally located bifurcations produced by *sgg/zw3* mutant clones distalize, compared to roughly half of the *wg*-produced bifurcations. A large proportion of either type of duplicated legs can distalize if the bifurcation occurs in distal leg segments. The subdivision of the tibia into proximal and distal regions is based on the spatial domain of *Distal-less* expression in the leg disc (B. Cohen, unpublished data).

Organizer activity depends on the position of ventral cells in the leg disc

We noted that the ability of clones of *sgg/zw3* mutant cells to reorganize dorsal-ventral pattern depends on the position of the clone in the leg. Unlike dorsally located clones, which lead to formation of duplicated legs by respecifying the fates of surrounding cells, *sgg/zw3* mutant clones in lateral positions cause simple outgrowths of leg. The outgrowths consist entirely of genetically marked *sgg/zw3* mutant cells, which adopt ventral-most identity (*n*=7; Fig. 5A). However, these clones do not reorganize pattern so as to incorporate wild-type cells into a duplicated leg. Careful examination of the organization of the leg in the vicinity of these clones suggests that the mutant cells do not affect the fate of the surrounding lateral cells. Clones of *wg*-expressing cells produce a comparable class of simple outgrowths which consist entirely of genetically marked ventrolateral cells (*n*=18; Fig. 5B). The *wg*-expressing ventrolateral cells are also unable to influence the fates of the surrounding cells. Since the fates of the mutant cells are inappropriate for their position in the leg, the clones are apparently unable to integrate into the normal pattern and the mutant cells are extruded from the leg as outgrowths.

These observations suggest that organizer activity is not a wholly autonomous property of ventral cells. Rather, organizer activity appears to depend on the context in which the clone of ventral cells is produced. Neither *wg*-expressing, or *sgg/zw3* mutant cells can cause fate respecification when the clones lie in the lateral region of the leg. Pattern respecification occurs only when the clones are located dorsally, near the endogenous anterior-posterior compartment boundary. The duplicated legs produced by both types of clones always bifurcate from the dorsal side of the endogenous leg (*n*=78 for bifurcations caused by *wg*-expressing clones, *n*=51 for bifurcations caused by *sgg/zw3* mutant clones; see Figs 3, 4 for examples). In a related experiment, Campbell et al. (1993) have observed that bifurcations caused by *wg*-expressing clones occur dorsally near the compartment boundary in the region where *decapentaplegic,* a *Drosophila* TGF-β homologue, is expressed. Taken together, these observations suggested that clones of ventral cells that cause bifurcations must be located dorsally near the anterior-posterior compartment boundary, while clones causing simple outgrowths are located laterally in either the anterior or posterior compartment, but not adjacent to the compartment boundary.
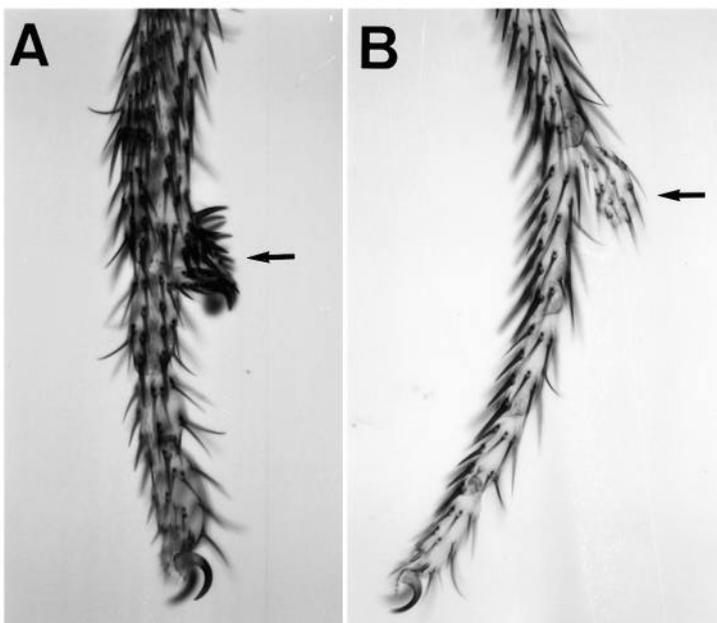

Fig. 5. Laterally positioned clones of ventral cells produce simple outgrowths of the leg. (A) Example of a clone of *sgg/zw3* mutant cells located at a lateral position in the second tarsal segment (arrow). The outgrowth consists entirely of genetically marked *sgg/zw3* mutant cells belonging to the clone (identified by *forked* mutant bristles). It is not clear whether the fusion of the first and second tarsal segments in this example is related to the clone. Unlike *sgg/zw3* mutant clones in dorsal positions, laterally located clones are unable to reorganize the pattern of the surrounding cells. Since the fates of the mutant cells are inappropriate for their position in the leg, these clones cannot integrate into the normal pattern and are therefore extruded from the leg as simple outgrowths. We have also observed clones of *sgg/zw3* mutant cells, which segregate internally to form vesicles (*n*=19; not shown). The vesicles are composed of mutant cells, which produce genetically marked ventral bristles projecting into the lumen. Since the vesicles are often detached from the leg, it is not possible to know where these clones of ventral cells originate; however, it is possible that they form in lateral positions and segregate internally. (B) Example of a clone of *wg*-expressing cells at a lateral position in the first tarsal segment (arrow). The outgrowth consists entirely of genetically marked cells belonging to the clone (identified by *yellow* bristles).

DISCUSSION

Organizing activity of ventral leg cells

Embryonic organizers have been identified in vertebrate embryos as groups of cells that can cause a global reorganization of spatial pattern when transplanted to an ectopic location. Cells from the dorsal lip of the blastopore of the amphibian embryo can organize an ectopic dorsal-ventral axis when transplanted to an ectopic position (Spemann and Mangold, 1924; Smith and Slack, 1983). Cells from the posterior margin of the chick limb bud reorganize anterior-posterior pattern when transplanted to the anterior margin of a host limb bud (Tickle et al., 1975). The cells that comprise an organizer are often thought of as the source of positional signals that exert a long-range influence on the fates of other cells (e.g. Smith and Harland, 1992; Tickle et al., 1982; Thaller and Eichele, 1987; Echelard et al., 1993; Krauss et al., 1993; Riddle et al., 1993). In *Drosophila*, groups of cells with organizer activity have been identified by production of genetic mosaics, which alter patterns of gene expression in situ, rather than by direct transplantation of cells from the organizer region (Struhl and Basler, 1993; Diaz-Benjumea and Cohen, 1993). Using this strategy, Struhl and Basler have shown that cells expressing the *wingless* (*wg*) gene can reorganize dorsal-ventral pattern in the adult leg of *Drosophila*. The ability of *wg*-expressing clones to respecify the fates of the surrounding cells was attributed to the fact that the Wg protein is secreted, and it was proposed that Wg acts as a concentration-dependent morphogen to specify positional information along the dorsal-ventral axis of the leg.

Although localized expression of *wg* is required to specify the ventral organizer, our results suggest that Wg does not function as a gradient morphogen. We have shown that clones of cells that lack the activity of the *sgg/zw3* gene possess ventral organizer activity indistinguishable from that of *wg*-expressing cells. Siegfried et al. (1992) have shown that Wg signaling in the embryo is mediated via inactivation of the serine-threonine kinase encoded by the *sgg/zw3* gene. Our results demonstrate that *sgg/zw3* serves as the downstream effector through which *wg* specifies ventral cell fate in the leg imaginal disc. Removing the activity of the sgg/zw3 kinase in a clone of cells is sufficient to specify the fate of those cells as ventral. The change in cell fate is cell autonomous, as expected since *sgg/zw3* encodes a cytoplasmic protein kinase (Bourouis et al., 1990; Siegfried et al., 1990); however, clones of *sgg/zw3* mutant cells are able to respecify the fates of surrounding wild-type cells in a non-autonomous manner. We conclude that organizer activity results from inactivation of the *sgg/zw3* kinase in ventral cells, independent of whether these cells secrete Wg protein. We can consider two models for how the *sgg/zw3* mutant cells influence the fates of the surrounding wild-type cells. It is possible that ventral cells serve as the source of some as yet unidentified secreted signaling molecule, which organizes pattern in a non-autonomous manner. However, for reasons outlined in the following section, we consider it more likely that ventral cells interact with dorsal cells to generate pattern. At present, we cannot distinguish between these possibilities.

Organizer activity depends on cell interaction

The ability of *sgg/zw3* mutant or *wg*-expressing cells to respecify the fates of their neighbors depends upon the context in which the clones of ventral cells are generated. Clones of ventral cells can elicit pattern reorganization and change the fates of the surrounding wild-type cells only when they are located in a dorsal position near the anterior-posterior compartment boundary. Campbell et al. (1993) have noted that bifurcations produced by *wg*-expressing clones originate in the dorsal region of the leg, close to the stripe of *decapentaplegic* (*dpp*) expression along the anterior-posterior compartment boundary. We have shown that ventral cells are apparently unable to interact with lateral cells to elicit pattern reorganization. Thus it appears that organizer activity depends on a combination of signals, one resulting from inactivation of *sgg/zw3*, the other possibly deriving from localized expression of *dpp*, as suggested by Campbell et al. (1993). Although it remains to be demonstrated that *dpp* expression constitutes the second signal, it is clear that proximity of cells with ventral identity to the anterior-posterior compartment boundary is required to elicit organizer activity.

Grafting experiments and the organizer

The experiments reported here, together with those of Struhl and Basler (1993) and Campbell et al. (1993) suggest an explanation for the observation that juxtaposition of dorsal and ventral tissues by grafting can lead to pattern reorganization of the cockroach leg (Bohn, 1965, 1972; French, 1976). Grafts between left and right limbs, combined with a 180° rotation of the donor limb causes a reversal of the dorsal-ventral axis of the graft, while retaining a normal anterior-posterior orientation with respect to the host limb (Fig. 6A). Bifurcations of the leg arise at both positions where host and graft tissues are mismatched, leading to triplication of the leg. As illustrated in Fig. 6, the orientation and symmetry of the triplicated legs produced by grafting is equivalent to that observed in the bifurcated legs caused by clones of *sgg/zw3* mutant cells. The supernumerary legs resulting from the grafts share a common plane of anterior-posterior symmetry. Following the reasoning used to explain the bifurcations produced by *sgg/zw3* mutant clones, we suggest that new organizers are generated by placing ventral cells in close proximity to dorsal cells near the presumed anterior-posterior compartment boundary of the cockroach leg. Since the graft generates two ectopic organizers, two supernumerary legs are formed.

Analogous experiments in which the anterior-posterior axes of graft and host legs were reversed, while retaining dorsal-ventral orientation also produced triplicated legs (performed by grafting right and left limbs, without a 180° rotation; Bohn, 1965, 1972; French, 1976). In this case, the supernumerary legs share a common axis of dorsal-ventral symmetry with the host leg. These grafts would produce ectopic anterior-posterior interfaces in proximity to the endogenous ventral cells, in effect the same combination shown above to elicit organizer activity. This interpretation is compatible with Meinhardt's (1983) boundary model, which suggests that organizing activity results from interaction between anterior-ventral cells, anterior-dorsal cells and posterior cells.

Evidence for two genetically distinct pathways for *wg* signaling

wg gene function is required for the organization of both the dorsal-ventral and proximal-distal axes of the leg. Mutations

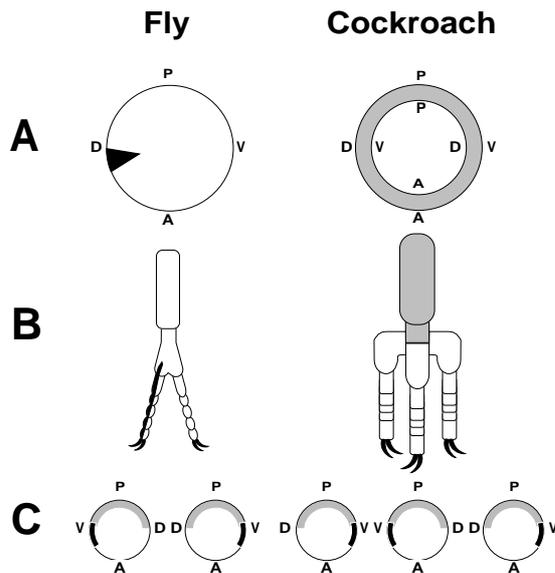**Fly**   **Cockroach**

Fig. 6. Grafting experiments in cockroach legs interpreted in terms of the ventral organizer. Grafting experiments performed on cockroach legs have shown that supernumerary limbs can be formed as a consequence of inappropriate juxtaposition of host and graft tissues following transplantation (Bohn, 1965, 1972; French, 1976). The ventral organizer model provides a reasonable explanation for these observations. (A) The experimental manipulation. Note: for simplicity in presentation of the structure of the adult legs, the diagrams are oriented so that the dorsal aspect of the host leg is to the left and posterior is up (V, ventral; D, dorsal; A, anterior; P, posterior). In *Drosophila*, a clone of ventral cells is generated in the dorsal region of the disc (e.g. mutant for *sgg/zw3*, depicted as a black sector. In cockroach, dorsal and ventral can be mismatched by grafting between left and right legs, coupled with a 180° rotation of the graft. The host limb is depicted as a filled circle (in the same orientation as the *Drosophila* disc). The grafted limb is depicted as an open circle. Note that dorsal and ventral are mismatched on both sides, while anterior and posterior are matched correctly. (B) The structure of the resulting legs. In *Drosophila*, a single supernumerary leg is generated. The bifurcation arises on the dorsal side of the host leg and the symmetry of the bifurcated leg is ventral out. The clone of ventral cells (black) runs as a stripe of cells in the ventral surface of the supernumerary leg. (C) Schematic representation of the arrangement of the ventral cells with respect to the anterior and posterior compartments in the endogenous and supernumerary legs (ventral cells are depicted by an arc of black shading, the posterior compartment is depicted by an arc of gray shading). In *Drosophila* the two ventral sides are in opposite polarity. In cockroach, one supernumerary limb is oriented with respect to the host leg (ventral to the right in diagram). The other supernumerary leg is oriented with respect to the graft (ventral to the left). Since the same population of ventral cells is interacting with dorsal cells near the anterior-posterior compartment boundary in graft and host tissue, the graft and supernumerary legs are oriented with the ventral side facing inward.

that reduce *wg* activity during larval development lead to the loss of ventral leg structures and to foreshortening of the leg along the proximal-distal axis (Baker, 1988; Peifer et al., 1991; Couso et al., 1993). Peifer et al. (1991) have shown that *armadillo* protein, a *Drosophila* homologue of β-catenin, is implicated in *wg*-dependent patterning of both the embryonic

segment and the dorsal-ventral axis of the leg. Reducing the level of *armadillo* gene activity produces phenotypes essentially identical to those obtained by reducing *wg* activity. Siegfried et al. (1992) have shown that the *sgg/zw3* kinase functions in the Wg signaling pathway in the embryonic segment, and we have shown here that all of the functions of *wg* in organizing dorsal-ventral axis of the leg can be explained by local inactivation of the *sgg/zw3* kinase. However, the requirement for *wg* in proximal-distal axis formation cannot be explained solely in terms of a signal mediated by inactivation of *sgg/zw3*. Our finding that duplicated legs associated with *wg*-expressing clones can distalize from proximal leg segments, while those associated with *sgg/zw3* mutant clones cannot, suggests that Wg can also act through a genetically distinct signaling pathway. Thus *wg* appears to play functionally distinct and separable roles in organizing pattern along the proximal-distal and dorsal-ventral axes of the leg.

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