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slimb coordinates *wg* and *dpp* expression in the dorsal-ventral and anterior-posterior axes during limb development

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SUMMARY

In the *Drosophila* leg disc, *wingless* (*wg*) and *decapentaplegic* (*dpp*) are expressed in a ventral-anterior and dorsal-anterior stripe of cells, respectively. This pattern of expression is essential for proper limb development. While the Hedgehog (Hh) pathway regulates *dpp* and *wg* expression in the anterior-posterior (A/P) axis, mechanisms specifying their expression in the dorsal-ventral (D/V) axis are not well understood. We present evidence that *slimb* mutant clones in the disc deregulate *wg* and *dpp* expression in the D/V axis. This suggests for the first time that their expression in the D/V axis is actively regulated during imaginal disc development. Furthermore, *slimb* is unique in that it also deregulates *wg* and *dpp* in the A/P axis. The misexpression phenotypes of *slimb*$^{-}$ clones indicate that the regulation of *wg* and *dpp* expression is coordinated in both axes, and that *slimb* plays an essential role in integrating A/P and D/V signals for proper patterning during development. Our genetic analysis further reveals that *slimb* intersects the A/P pathway upstream of *smoothened* (*smo*).

Key words: *Drosophila*, Axial patterning, Leg disc, *wingless*, *decapentaplegic*, Cell signaling

INTRODUCTION

Cells in *Drosophila* imaginal discs proliferate and organize in larval and pupal stages to form adult structures with specific patterns. Several secreted factors are responsible for coordinating the precise patterning of imaginal tissues for proper limb development. Hh, expressed in posterior cells induces neighboring anterior compartment cells to express their own anterior determinants: the *Drosophila* TGFβ homolog *decapentaplegic* (*dpp*), and the Wnt family member *wingless* (*wg*) (Lee et al., 1992; Basler and Struhl, 1994; Capdevila et al., 1994; Tabata and Kornberg, 1994; Felsenfeld and Kennison, 1995). In the leg imaginal disc, expression of *dpp* in the dorsal-anterior stripe is required for specification of dorsal structures, while *wg* in the ventral-anterior stripe determines ventral structures (Ferguson and Anderson, 1992; Struhl and Basler, 1993; Wilder and Perrimon, 1995). The expression patterns of *dpp* and *wg* are defined in both the anterior-posterior (A/P) and dorsal-ventral (D/V) axes.

The restricted domains of *dpp* and *wg* expression are tightly regulated in the A/P axis by the Hh/Ptc and PKA signaling pathways (Phillips et al., 1990; Ingham et al., 1991; Basler and Struhl, 1994; Capdevila and Guerrero, 1994; Tabata and Kornberg, 1994; Felsenfeld and Kennison, 1995; Li et al., 1995). Inactivation of *ptc* and *pka* or ectopic expression of *hh* induces ectopic *dpp* expression in the dorsal-anterior and ectopic *wg* expression in the ventral-anterior of the leg disc (Phillips et al., 1990; Ingham et al., 1991; Basler and Struhl, 1994; Jiang and Struhl, 1995; Li et al., 1995; Pan and Rubin, 1995) (Capdevila et al., 1994; Lepage et al., 1995). However, mutations in components of the A/P signaling pathway do not alter *wg* and *dpp* expression patterns in the D/V axis. To date no evidence exists of a D/V signaling pathway. It is possible that the D/V axis defined during embryogenesis is retained in imaginal tissues. One mechanism that prevents misexpression of *wg* in the dorsal and *dpp* in the ventral is the antagonistic relationship between *wg* and *dpp*. Inactivation of Wg or Dpp signaling leads to ectopic expression of *dpp* or *wg*, respectively (Brook and Cohen, 1996; Jiang and Struhl, 1996; Penton and Hoffman, 1996; Theisen et al., 1996).

To identify recessive overproliferation mutations in genes which are lethal in homozygous mutant animals, we have performed genetic screens in mosaic flies containing homozygous mutant patches in otherwise wild-type backgrounds (Xu et al., 1995). Two classes of recessive overproliferation mutations have been identified. Mutations of the first group cause mutant cells to undergo extensive proliferation and form unpatterned, tumorous outgrowths in mosaic adults. Mutations of the second group induce both patterned and irregular outgrowths. Here we report a new gene of the second class, *slimb*, which affects developmental signals that regulate cell proliferation and pattern organization. We present evidence that *slimb* mutant cells induce outgrowths by misexpressing *wg* and *dpp*. *slimb* regulates *wg* and *dpp* in both the A/P and D/V axes,

demonstrating for the first time that these signals are coordinated. Genetic epistasis experiments reveal that *slimb* intersects A/P signaling upstream of *smo*.

MATERIALS AND METHODS

slimb fly strains

122 excision lines were generated from two P-alleles ($slmb^{00295}$ and $slmb^{05415}$) and about half of them reverted to wild type. More than 30 excision alleles behaved as a single complementation group. Strong *slmb* alleles, including the original P alleles and $slmb^{e4\text{-}1}$ (Fig. 2A), caused embryonic lethality while weak alleles caused larval and pupal lethality.

Cloning of *slimb* and H-*slimb*

Genomic DNA surrounding the P-insertion sites was obtained by plasmid rescue and used to isolate a genomic cosmid and cDNAs from an imaginal disc library. Comparison of genomic and cDNA sequences showed that $slmb^{00295}$and $slmb^{05415}$ inserted 150 nucleotides upstream of and within the coding region, respectively. Southern blot analysis of genomic DNA generated from the excision lines revealed that $slmb^{e4\text{-}1}$ carries an approx. 3 kb deletion removing the 5′ end of the *slmb* transcript. The two P-alleles behave similarly to $slmb^{e4\text{-}1}$ and are used interchangeably, while other excision alleles have weaker phenotypes. The 3.5 kb cDNA was sequenced to predict a protein product and cloned into the pCaSpeR-hs vector for germline transformation. Three of the transformant lines were able to fully rescue the lethality of the amorphic *slmb* alleles after 1 hour of heat shock every 24 hours during larval and pupal development. The human *slmb* homolog was identified by using the *Drosophila* cDNA as a probe to screen a human fetal brain library.

Generation and analysis of clones

Clones in adult flies and imaginal discs were generated by FLP-mediated mitotic recombination as previously described (Xu and Rubin, 1993; Xu and Harrison, 1994). Eggs from the appropriate crosses were collected for 24 hours and cultured at 25°C. Clones were induced in early second instar larvae by heat-shock induction of Flipase (38°C for 1 hour). Larvae from the following genotypes were used for clonal analysis: *yw hsFLP1; P[FRT]82B P[πM]87E* Sb^{63b} $P[y^{+}]96E/P[FRT]82B$ $slmb^{e4\text{-}1\ or\ 00295}$ in a *H1-1dpp-lacZ*/+ background; and in a *wg-lacZ*/+ background. To detect *hh-lacZ* expression in *slmb* clones, *hh-lacZ-P30* was recombined onto the *slmb* mutant chromosome and clones were induced in the following larvae: *yw hsFLP1; P[FRT]82B P[πM]87E hh-lacZ-P30 P[πM]97E /P[FRT]82B* $slmb^{00295}$ *hh-lacZ-P30*. Staining procedures followed standard protocols (Xu and Harrison, 1994).

Double mutant clones were induced in flies homozygous for the *slmb* null allele, but carried the *hs-slmb31* rescue construct on the FRT40A chromosome arm. To ensure the full rescue of $slmb^{-}$ flies, eggs were collected every 24 hours and heat-shocked daily at 38°C for 60 minutes until hatched. Larvae of the following genotypes were generated and cultured at 25°C: *yw hsFLP1; hs-slmb 31 P[FRT]40A/*wg^{CX4} *ck P[FRT]40A;* $slmb^{00295}/slmb^{00295}$, *yw hsFLP1; hs-slmb 31 P[FRT]40A/*dpp^{12} *ck P[FRT]40A;* $slmb^{00295}/slmb^{00295}$, *yw hsFLP1; P[FRT]82B P[πM]87E* Sb^{63b} $P[y^{+}]96E$ *X/P[FRT]82B* $slmb^{00295}$ hh^{rJ413}, and *yw hsFLP1; hs-slmb 31 P[FRT]40A/*smo^{D16} *ck P[FRT]40A;* $slmb^{00295}/slmb^{00295}$. $slmb^{-}$ clones were induced using the *hs-slmb 31 P[FRT]40A* chromosome at a frequency of 60% of discs. To verify that the smo^{D16} *ck P[FRT]40A* chromosome that we used did not cause a cell-lethal phenotype, we examined clonal production by this chromosome and found it to produce smo^{-} clones at a frequency of more than 25% of discs. In analyzing smo^{D16}, $slmb^{00295}$ double mutant clones, 40 mosaic leg discs were stained and found to have no ectopic *wg* or *dpp* expressed.

RESULTS AND DISCUSSION

In a mosaic screen to identify recessive overproliferation mutations, we identified a new mutation, *shiva*, which causes outgrowths and disrupts pattern formation (Fig. 1) (Xu et al., 1995). In addition to two original P-insertion alleles, a deletion null allele ($shiva^{e4\text{-}1}$) was generated by excision of

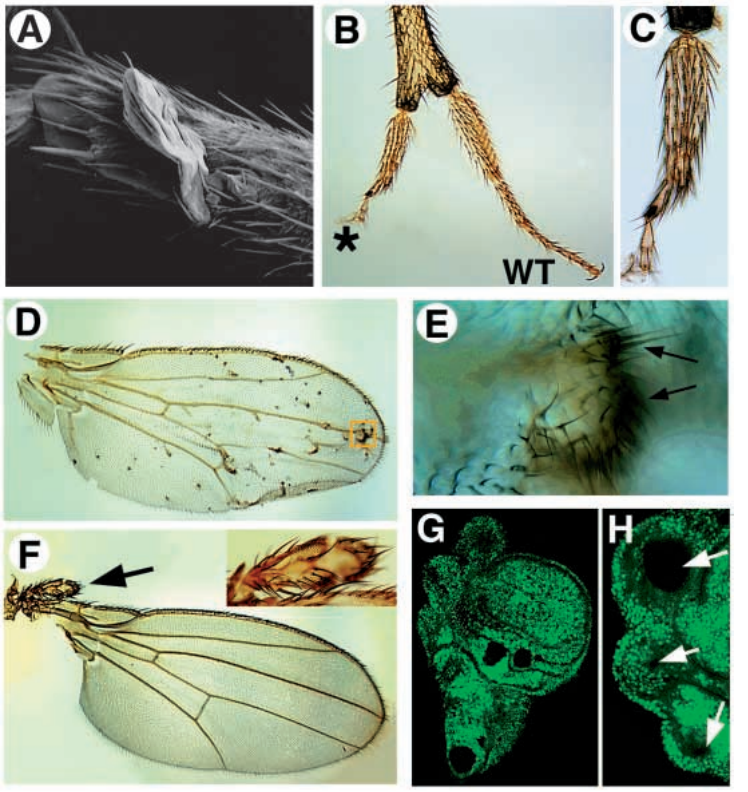

Fig. 1. *slimb* clonal phenotypes in adult limbs. The *yellow*$^{-}$ (y^{-}) and *Stubble* (*Sb*) cuticle markers were used to label $slmb^{e4\text{-}1}$mutant cells in adult flies. Two types of outgrowths were observed in *slmb* mosaic legs: simple tissue outgrowths (A) and duplicated structures (B). (A) Scanning electron micrograph shows a leg tissue outgrowth composed of $slmb^{+}$ cells (y^{+}, *Sb* cells, y^{+} cannot be visualized on SEM). (B) Bifurcation of a third leg. The endogenous third leg (right) has signature posterior transverse and ventral bristles. The ectopic limb (asterisk) has $slmb^{+}$ (y^{+}, *Sb*) bristles, which are only of the dorsal type (close-up in C). As with the leg, wings from $slmb^{e4\text{-}1}$ mosaic animals also displayed two types of outgrowths (D,F). (D) A *slimb* mosaic wing blade with multiple outgrowths. Outgrowths are found on either the dorsal or ventral surface of the blade, and in both anterior and posterior regions. (E) Magnification of an outgrowth indicated by the box in D. The outgrowth spans both sides of the third wing vein, and contains second row bristles at its apex (arrows). (F) A supernumerary wing (arrow) extends from the wing hinge-region and contains near-mirror images of anterior-most patterns and is composed of y^{+} cells (inset). (G) A $slmb^{P00295}$ mosaic wing disc has extensive outgrowths. (H) High magnification of outgrowths in G. $slimb^{-}$ clones are located at the center of these outgrowths (H, arrows), inducing surrounding wild-type cells to proliferate.

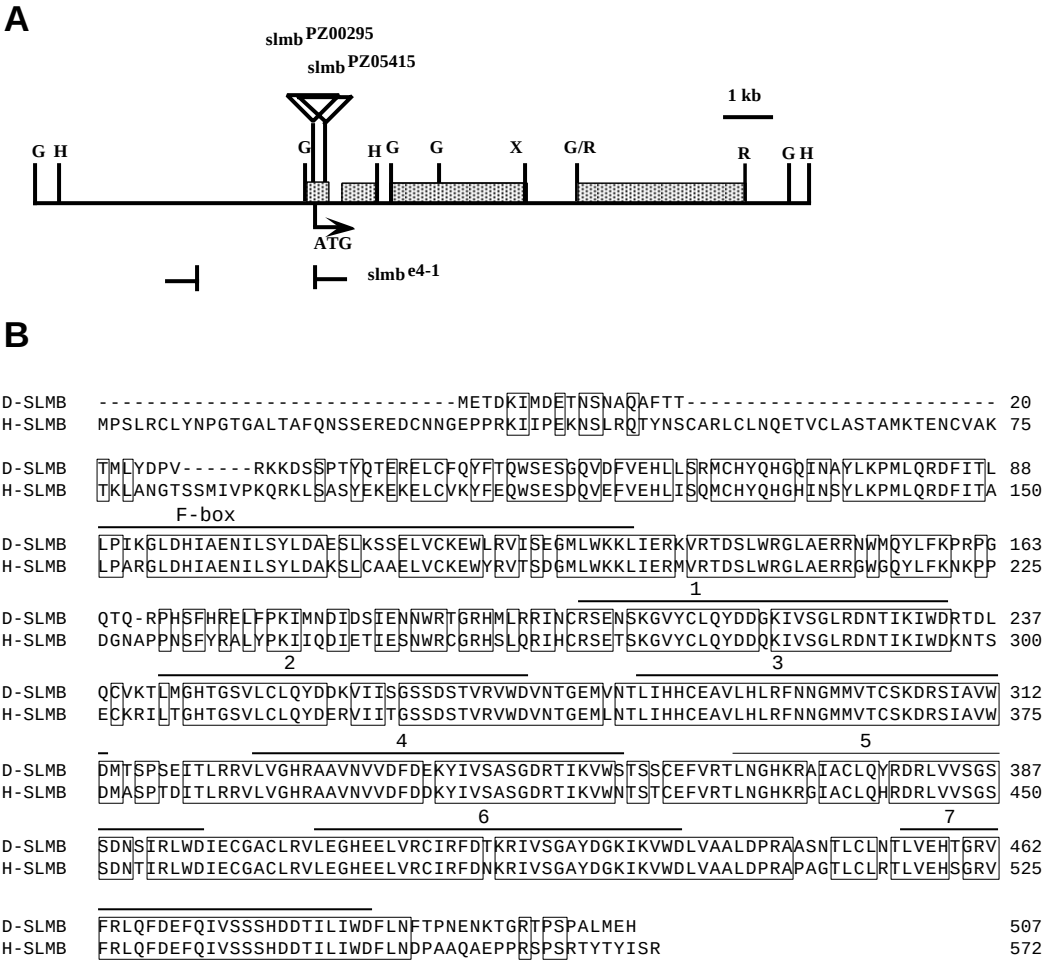

Fig. 2. The *slimb* gene and its homologs. (A) The *slimb* transcript is illustrated on the genomic restriction map. An arrow indicates the initiation codon and direction of transcription. Hatched boxes indicate exon regions. The *slmb* P-alleles (*slmb*^{00295} and *slmb*^{05415}) have P-elements in the first exon, and the excision line *slmb*$^{e4\text{-}1}$ deletes the *slmb* promoter and transcript regions. Restrictions sites: G, BglII, H, *Hin*dIII; X, *Xba*I; R, *Eco*RI. (B) The *Drosophila* and human *slmb* transcripts predict protein products which share extensive homology throughout (boxes). Accession no. AF032878.

the P-elements and used for phenotypic analysis (Fig. 2A). Molecular and genetic characterization of *shiva* reveals that these mutations disrupt a single transcriptional unit and they can be rescued when the cDNA is expressed under control of the heat shock-inducible promoter (Materials and Methods). The transcript encodes a Cdc4-related protein containing F-box and WD-40 motifs. During preparation of this manuscript, Jiang and Struhl independently reported the identification of this gene as *slimb* (Jiang and Struhl, 1998). Thus, we are now renaming our gene *slimb*. Using a *Drosophila slimb* cDNA, we also isolated a human homolog (H-*slimb*) (Fig. 2B). The fly and human proteins share 78% amino acid identity throughout, suggesting that *slimb* is functionally conserved.

slimb induces outgrowths in mosaic adults

Phenotypic analysis revealed that *slmb*$^{-}$ clones induce tissue outgrowths and supernumerary limbs in mosaic adults (Fig. 1). To analyze the *slmb* mosaic phenotype, the *yellow*$^{-}$ (*y*$^{-}$) and *Stubble*$^{+}$ (*Sb*$^{+}$) cuticular markers were used to label *slmb*$^{-}$ cells (Xu and Rubin, 1993; Materials and Methods). In mosaic legs, outgrowths are composed of *slmb*$^{+}$ cells (*y*$^{+}$ and *Sb*) (Fig. 1A-C). In addition to irregular outgrowths, supernumerary legs derived from *slmb*$^{+}$ cells are also observed in *slmb* mosaic animals (Fig. 1B,C). Outgrowths are also observed in the wing blade (Fig. 1D,F), where mutant clones for *slmb* frequently produced small outgrowths in the adult wing (Fig. 1D). Similar to the leg outgrowths, the wing outgrowths consist of *slmb*$^{+}$ cells (*y*$^{+}$ and *Sb*) (Fig. 1E). Moreover, these outgrowths project from both the ventral and dorsal surfaces of the blade and occur in both the anterior and posterior halves of the wing (Fig. 1D). Outgrowths were organized into wing blade-like structures with wing margin bristles normally seen at the corresponding wing margin (Fig. 1D,E). Rarely, supernumerary wings consisting of symmetric duplications of anterior-most structures develop at the wing hinge (Fig. 1F). Although *y*$^{-}$ (*slmb*$^{-}$) cells are rarely observed in mosaic animals, examination of mosaic discs revealed overproliferation of wild-type cells surrounding *slmb* mutant clones (Fig. 1G,H). These observations lead us to conclude that the mutant cells did not survive to adult stage, and that the adult outgrowths they induced are vestiges of their presence.

slimb regulates *wg* and *dpp* expression in both the A/P and D/V axes of the leg disc

slmb-induced outgrowths are reminiscent of the phenotypes caused by misexpression of *dpp* and *wg* (Struhl and Basler, 1993; Basler and Struhl, 1994; Wilder and Perrimon, 1995). Thus, we examined *dpp* and *wg* expression in *slmb* mosaic leg discs using *wg-lacZ* and *dpp-lacZ* reporter genes (Blackman et al., 1991; Kassis et al., 1992). *slmb* clones ectopically express both *wg* and *dpp* in a cell-autonomous fashion (Fig. 3). In

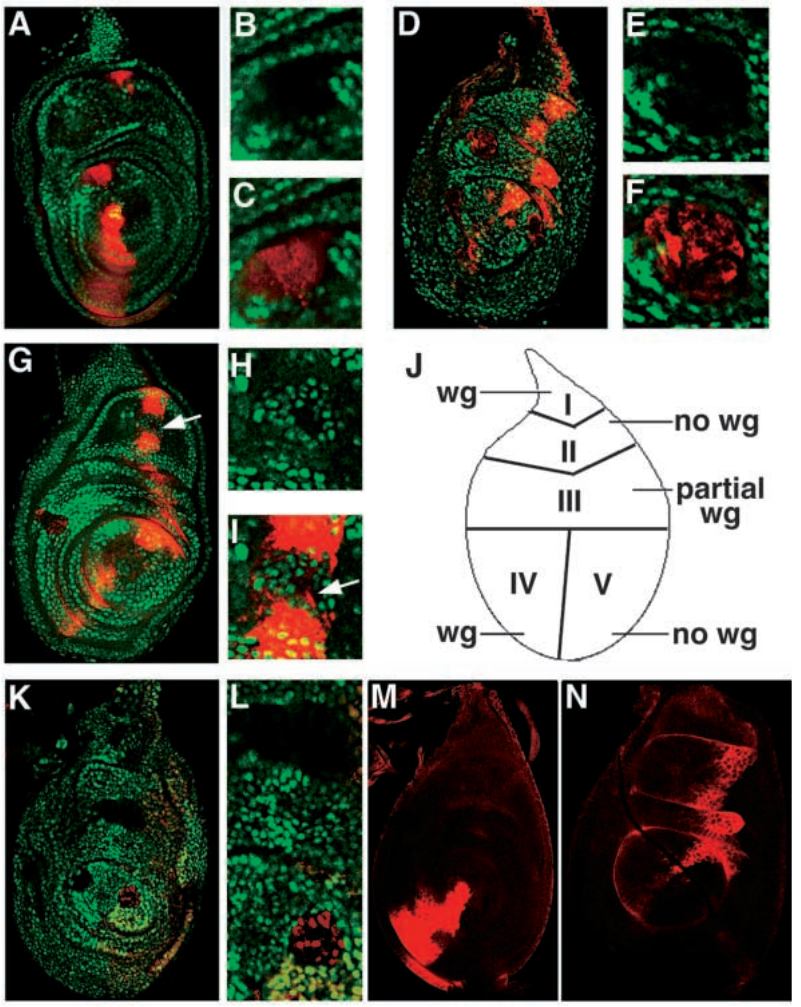

Fig. 3. *slimb* induces ectopic expression of *wg* and *dpp* in leg imaginal discs. In all panels, third instar leg discs are positioned with anterior to the left and ventral down. $slmb^{e4\text{-}1}$ and $slmb^{P00295}$ clones are marked by the absence of anti-Myc staining (green). *wg-lacZ* and *dpp-lacZ* and hh^{P30} expression patterns were visualized with anti-β-gal antibody (red). (A) Leg disc bearing $slmb^{P00295}$ clones in the dorsal region associated with ectopic *wg-lacZ* expression. (B,C) Close-up images of the clone in A illustrating $slmb^{-}$ clone (lacking green; B) with ectopic *wg* expression (red; C). (D) $slmb^{e4\text{-}1}$ clones also ectopically express *dpp*. (E,F) Close-up images of the clone in D illustrating a $slmb^{-}$ clone (lacking green; E) with ectopic *dpp* expression (red; F). (G) $slmb^{e4\text{-}1}$ clones in the endogenous domain for *dpp* expression (arrow). (H,I) Close-up images of the clone indicated by an arrow in G, illustrating a $slmb^{-}$ clone (lacking green, H) in which *dpp* is expressed in the $slmb^{-}$ cells, but is suppressed in nearby wild-type cells (I, arrow). (J) Diagram illustrating *wg* expression based on 103 analyzed $slmb^{-}$ clones; five subregions are apparent. At the dorsal tip (I) and ventral-anterior (IV) regions, *wg* is ectopically expressed in all $slmb^{-}$ cells of a clone. Region III, however, which spans the D/V border is unique in that only a fraction of the mutant cells ectopically express *wg*. No ectopic *wg* expression has been observed in regions II and V. *dpp* expression in 98 $slmb^{-}$ clones was analyzed and not found to fall into any distinct domains. (K) In contrast to their effects on *wg* and *dpp* expression, $slmb^{P00295}$ clones do not alter *hh* expression. (L) Close-up images of the clone in K illustrating the anterior $slmb^{-}$ clone (top) does not express *hh*. (M,N) smo^{D16}, $slmb^{P00295}$ double mutant mosaic leg discs express no ectopic *wg* (M) or *dpp* (N).

respect to the A/P regions, 58/72 A clones and 9/31 P clones ectopically expressed *wg*, and 43/81 A clones and 6/17 P clones ectopically expressed *dpp*. A composite view of *wg* expression in the 103 analyzed $slmb^{-}$ clones are illustrated in five subregions (Fig. 3J). *dpp* expression of 98 $slmb^{-}$ clones was analyzed and not found to fall into any distinct domains. *slmb* mutant clones deregulate *wg* and *dpp* in both D/V and A/P axes. Ectopic *wg* expression is observed in both ventral and dorsal regions (Fig. 3A-C,J). Similar results are also observed for *dpp* (Fig. 3D-I). In *slmb* mutant clones situated within or near the endogenous *dpp* expression zone, *dpp* was expressed in the mutant cells but down-regulated in adjacent wild-type cells (Fig. 3G-I). Previously it had been shown that Wg and Dpp signaling mutually antagonize each other's expression, which prevents expression of the two molecules in the same cells (Brook and Cohen, 1996; Jiang and Struhl, 1996; Penton and Hoffman, 1996; Theisen et al., 1996). Ectopic expression of both *wg* and *dpp* in $slmb^{-}$ clones in the dorsal-anterior of the leg disc indicates a disruption of this mutual antagonism.

Although *lacZ* reporter genes may not always reflect protein expression, these reporter genes have been previously shown to serve as faithful indicators for *wg* and *dpp* gene expression in the leg disc (Jiang and Struhl, 1995; Li et al., 1995; Brook and Cohen, 1996; Jiang and Struhl, 1996; Penton and Hoffman, 1996; Lecuit and Cohen, 1997). To test whether ectopic *wg* and *dpp* expression are responsible for the outgrowth phenotype in *slmb* mosaic animals, we generated flies carrying clones of cells mutant for both *slmb* and *wg*, or *slmb* and *dpp*. In comparison to *slmb* mutant clones, double mutant clones do not cause any significant outgrowths (Table 1). Therefore, Wg and Dpp are two primary effector molecules responsible for the induction of outgrowths in *slmb* mosaic animals. These results are consistent with previous observations that *wg* and *dpp* are both required for defining the proximodistal outgrowth center (Diaz-Benjumea et al., 1994; Campbell and Tomlinson, 1995; Lecuit and Cohen, 1997).

slimb coordinates D/V and A/P signals to specify *wg* and *dpp* expression patterns

The *slmb* phenotype differs from those of all previously known genes, in that it is the first gene found to deregulate both *wg* and *dpp* expression in the D/V axis. Disrupting components of the Hh signaling pathway deregulates *wg* and *dpp* only along the A/P axis. For example, ectopic activation of *hh* or removal of *ptc* and *pka* results in misexpression of *dpp* and *wg* in anterior cells that normally do not express these genes. However, *wg* misexpression is always restricted to the ventral

Table 1. Double mutant clone analysis

Clonal genotype	Number of flies with leg outgrowths
slmb	65/96
wg, slmb	1/150
dpp, slmb	3/118
smo, slmb	3/114
hh, slmb	97/126

cells, while *dpp* misexpression is only in dorsal cells (Basler and Struhl, 1994; Jiang and Struhl, 1995; Li et al., 1995; Pan and Rubin, 1995). Thus, the control of *wg* and *dpp* expression in the D/V axis is not disrupted. The mechanism restricting *wg* and *dpp* in the D/V axis is not known. It is possible that the ability of dorsal cells to express *dpp* and of ventral cells to express *wg* is an inherent property of the D/V identity established during embryogenesis. The mutant phenotype of *slmb*$^{-}$ clones in discs provides the first evidence that *wg* and *dpp* expression in the D/V axis is actively regulated during imaginal disc development, and is not solely defined during embryonic development. Since the Hh pathway regulates *wg* and *dpp* expression in the A/P axis, our results suggest that a pathway different from Hh may operate in imaginal discs to restrict their expression in the D/V axis (Fig. 4). This pathway cannot be either the Wg or Dpp signaling pathway since inactivation of Wg or Dpp signaling affects either *dpp* or *wg* expression, but not both (Brook and Cohen, 1996; Jiang and Struhl, 1996; Penton and Hoffman, 1996; Theisen et al., 1996). The *slmb* phenotypes described here were not observed in the previous study which used weak *slmb* alleles and revealed only A/P defects (Jiang and Struhl, 1998). The phenotypic differences probably reflect the fact that we have used a null allele instead of hypermorphic alleles.

In addition to D/V defects, *slmb* mutant clones also deregulate *wg* and *dpp* expression in the A/P axis. *slmb* is the first identified gene that regulates both *wg* and *dpp* expression in the A/P as well as D/V axes. The fact that mutations in *slmb* affect patterning in both axes suggests that the A/P and D/V signals are coordinated to specify *wg* and *dpp* expression patterns, and that *slmb* plays an essential role in integrating these signals (Fig. 4).

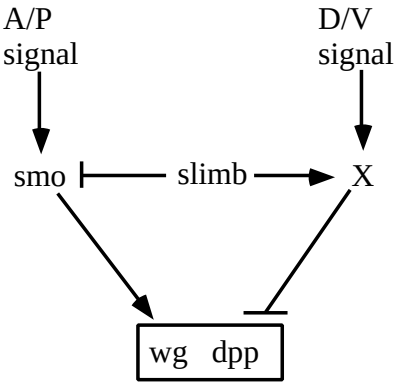

Fig. 4. A model for *slmb* function. *slmb* acts upstream of *smo* in A/P signaling which induces *wg* and *dpp* expression, and also participates in an unknown D/V signaling pathway (X) which restricts *wg* and *dpp* expression. Inactivation of *slmb* deregulates *wg* and *dpp* expression in both A/P and D/V axes.

slimb intersects A/P signaling upstream of *smo*

To further explore how *slmb* regulation and function correlates with A/P signaling, we carried out double mutant analysis with *slmb* mutants and with mutants of *hh* and *smo*. No reduction of outgrowths was observed in *slmb*$^{-}$, *hh*$^{-}$ double mutant clones (Table 1). Furthermore, *slmb* mutant clones have no effect on *hh* expression (Fig. 3K,L). This indicates that *slmb* acts downstream or independent of Hh signaling. In contrast, *slmb*$^{-}$, *smo*$^{-}$ double mutant clones almost completely suppress *slmb* induced outgrowths (Table 1). Consistent with the adult phenotype, discs carrying *slmb*$^{-}$, *smo*$^{-}$ clones fail to ectopically express either *dpp* or *wg* (Fig. 3M,N). These data suggest that *slmb* intersects the A/P signal upstream of *smo* (Fig. 4). The previous study suggested that *slmb* acts downstream of *smo* (Jiang and Struhl, 1998). This difference may be explained by the use of different alleles for *smo* and *slmb*. Many *smo* mutations are hypermorphic alleles which produce variable phenotypes (Alcedo et al., 1996; Heuvel and Ingham, 1996). *smo*^{D16} used in our analysis is caused by a DNA rearrangement which disrupts the *smo* transcript and produces the most severe embryonic phenotype (Alcedo et al., 1996; Heuvel and Ingham, 1996). The *slmb* product contains WD-40 repeats believed to act as a scaffold for the binding of multiple proteins (Neer et al., 1994; Sondek et al., 1996; Feldman et al., 1997; Skowyra et al., 1997). It is possible that this structure may allow for proteins such as smo and components of a D/V pathway to converge. The Slmb-related protein Cdc4 from *Saccharomyces cerevisiae* along with Cdc53, and Cdc34 are part of the ubiquitin proteolysis machinery (Yochem and Byers, 1987; Goebl et al., 1988; Bai et al., 1996; Willems et al., 1996). Our data that Slmb acts upstream of Smo, together with its sequence homology with Cdc4, suggests that Slmb could be involved in the regulation of Smo protein degradation.

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