

## Requirements of *Lim1*, a *Drosophila* LIM-homeobox gene, for normal leg and antennal development

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### SUMMARY

During *Drosophila* leg development, the distal-most compartment (pretarsus) and its immediate neighbour (tarsal segment 5) are specified by a pretarsus-specific homeobox gene, *aristaless*, and tarsal-segment-specific *Bar* homeobox genes, respectively; the pretarsus/tarsal-segment boundary is formed by antagonistic interactions between *Bar* and pretarsus-specific genes that include *aristaless* (Kojima, T., Sato, M. and Saigo, K. (2000) *Development* 127, 769–778). Here, we show that *Drosophila* *Lim1*, a homologue of vertebrate *Lim1* encoding a LIM-homeodomain protein, is involved in pretarsus specification and boundary formation through its activation of *aristaless*. Ectopic expression of *Lim1* caused *aristaless* misexpression,

while *aristaless* expression was significantly reduced in *Lim1*-null mutant clones. Pretarsus *Lim1* expression was negatively regulated by *Bar* and abolished in leg discs lacking *aristaless* activity, which was associated with strong *Bar* misexpression in the presumptive pretarsus. No *Lim1* misexpression occurred upon *aristaless* misexpression. The concerted function of *Lim1* and *aristaless* was required to maintain Fasciclin 2 expression in border cells and form a smooth pretarsus/tarsal-segment boundary. *Lim1* was also required for femur, coxa and antennal development.

Key words: Leg development, Homeobox genes, LIM-homeobox genes, *BarH1*, *BarH2*, *Fas2*, *aristaless*, *Lim1*, *Xlim1*, *Drosophila*

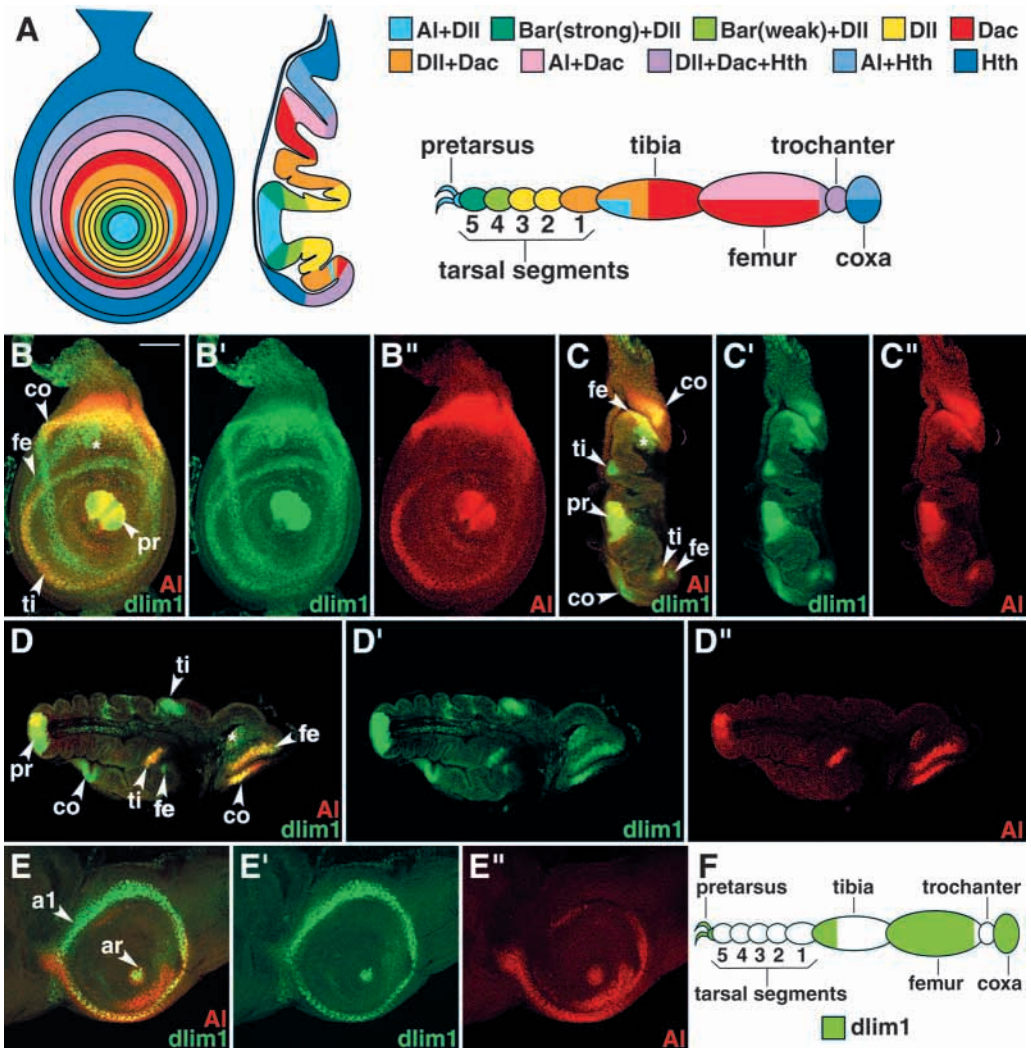
### INTRODUCTION

Vertebrate limbs and invertebrate appendages are formed through subdivision of the corresponding developing field and each subdomain, possibly with its own particular properties such as specificity in local cell adhesivity, may be specified by a combinatorial region-specific expression of transcription factors. Thus, it is important to clarify the manner in which transcription factor expression domains are generated and the mechanisms by which they determine local fates of developing limbs or appendages.

*Drosophila* adult leg consists of several segmental units, which, in proximal-distal direction, are the coxa, trochanter, femur, tibia, tarsal segments 1–5 and pretarsus – the latter bearing claws, pulvilli and an empodium. These segments develop through concentric subdivision of the leg disc epithelium, a mono-layered cell sheet that invaginates from the epidermis during embryogenesis. Distal segments are derivatives of the central region of the leg disc, while proximal segments, derivatives of the peripheral region. The concentric subdivision of the disc epithelium occurs in multiple phases. At the earliest stages of leg disc development, disc epithelium is divided into a distal region expressing *Distal-less* (*Dll*) and a proximal region expressing

*homothorax* (*hth*), *escargot* (*esg*) and *teashirt* (*tsh*) (Abu-Shaar and Mann, 1998; Erkner et al., 1999; Goto and Hayashi, 1999; Wu and Cohen, 1999). *Dll* and *hth* are homeobox genes (Cohen et al., 1989; Rieckhof et al., 1997), while *esg* and *tsh* encode zinc-finger proteins (Fasano et al., 1991; Whiteley et al., 1992; Fuse et al., 1994). As development proceeds, these domains undergo further subdivision into smaller domains through the action of *dachshund* (*dac*), encoding a novel nuclear factor (Mardon et al., 1994; Abu-Shaar and Mann, 1998; Wu and Cohen, 1999), and finally, into the regions corresponding to adult leg segments or components (Fig. 1A). Regulatory interactions between transcription factor genes expressed in neighbouring domains have been implicated to be essential for precise subdomain determination (Abu-Shaar and Mann, 1998; Erkner et al., 1999; Wu and Cohen, 1999; Kojima et al., 2000).

*BarH1* and *BarH2*, a pair of homeobox genes at the *Bar* locus (Kojima et al., 1991; Higashijima et al., 1992a), are essential for distal leg segmentation and specification of tarsal segments 3–5 in a functionally redundant manner (Kojima et al., 2000); they are hereafter collectively referred to as *Bar*. *aristaless* (*al*) is a homeobox gene expressed in the distal-most segment, pretarsus, and required for normal pretarsus



**Fig. 1.** Expression pattern of *Lim1* (*dlim1*) and other genes in leg and antennal discs. Dorsal is towards the top in all figures. (A) The expression pattern of *al*, *Bar*, *DII*, *dac* and *hth* in late third instar discs (left) and their extrapolated pattern in the adult leg (right) are shown schematically. The right-hand disc represents a sagittal section. (B-E'') Late third instar leg discs (B-C''), early pupal legs (D-D'') or late third instar antennal discs (E-E'') were stained for *Lim1-lacZ* (green) and AL (red). Merged images are shown in (B,C,D,E), where overlapping signals appear yellow. (C-D'') are sagittal optical sections. pr, ti, fe, co, ar and a1, respectively, show *Lim1-lacZ* expression in the pretarsus, tibia, femur, coxa, arista and first antennal segment. Asterisk indicates *Lim1-lacZ* expression in developing sensory organ precursors of the femoral chordotonal organ. (F) Locations of adult leg cells that expressed *Lim1* at late third instar. Scale bar: 50  $\mu$ m.

development (Campbell et al., 1993; Schneitz et al., 1993; Campbell and Tomlinson, 1998). *al* and *Bar* expression domains are initially determined as broad domains that partially overlap each other but at slightly later stages, a line of demarcation between *al* and *Bar* expression domains becomes evident through auto-regulation of *Bar* and mutually exclusive interactions between *Bar* and pretarsus factors (Kojima et al., 2000). Although *al* is included in pretarsus factors, other pretarsus genes may also be necessary for effective repression of *Bar*. Indeed, little or no reduction in *Bar* expression could be detected in future tarsal segments 4 and 5 following *al* misexpression, while *Bar* misexpression brought about considerable reduction in *al* expression in the prospective pretarsus region (Kojima et al., 2000).

Here, we show that *Lim1*, which encodes a protein similar in sequence to a vertebrate LIM-homeodomain protein, LIM1 (Taira et al., 1992; Fujii et al., 1994; Barnes et al., 1994), serves as a pretarsus element. Our results also indicated that, in the future pretarsus, *al* expression is positively regulated by LIM1, while *Lim1* expression is under the negative control of *Bar*. In addition, *Lim1* was found to be required not only for pretarsus development but the formation of femur, coxa and antennal structures as well.

## MATERIALS AND METHODS

### Fly strains

Flies used in this study were raised on standard medium at 25°C. Fly strains used are Canton-S (wild-type), *ptc*-GAL4 (559.1; Hinz et al., 1994), *blk*-GAL4 (40C.6; Morimura, et al., 1996), *UAS-BarH1<sup>M6</sup>* (Sato et al., 1999b), and *UAS-al<sup>1</sup>* (Kojima et al., 2000), *al<sup>1</sup>*, *alice* and *al<sup>ex</sup>* (Campbell and Tomlinson, 1998). *al<sup>ex</sup>* and *alice* are a null and a strong hypomorphic mutant, respectively. *alice/al<sup>ex</sup>* flies exhibit the same leg and antennal phenotypes as *al<sup>ex</sup>* mosaic clones (Campbell and Tomlinson, 1998) and no detectable level of AL was observed by immunostaining in *alice/al<sup>ex</sup>* leg and antennal discs (data not shown). Thus, the genotype of *alice/al<sup>ex</sup>* are referred to as *al<sup>ex</sup>* in this paper. P0092 (*Lim1-lacZ*) was obtained from FlyVie (http://pbio07.uni-muenster.de/). *UAS-Lim1* was generated by inserting an *EcoRI-XhoI* fragment of GH04929 (Berkeley Drosophila Genome project (BDGP; http://fruitfly.berkeley.edu/)) into pUAST (Brand and Perrimon, 1993). For FRT/FLP mosaic analyses, *FRT19A* (Xu and Rubin, 1993) and *eyFLP5* (Newsome et al., 2000) were used.

### FRT/FLP mosaic analysis

*Lim1<sup>-</sup>* clones were generated in larvae whose genotype are *Lim1<sup>7B2</sup> FRT19A/y w arm-lacZ FRT19A; eyFLP5/+*. *eyFLP5* seems to express *FLPase* also in the leg and antennal discs from early stages of

development and can induce mosaic clones before the onset of third instar.

**Ectopic expression of *Bar*, *al* and *Lim1***

Both *ptc*-GAL4 and *blk*-GAL4 can drive gene expression along the anterior-posterior (A/P) compartment boundary (Hinz et al., 1994; Morimura, et al., 1996). In most experiments, UAS-*Bar*<sup>H1M6</sup> and UAS-*al* were driven by *ptc*-GAL4. Any appreciable mutant phenotype was given by UAS-*al* driven by neither *ptc*-GAL4 nor *blk*-GAL4, while *blk*-GAL4-driven UAS-*Bar* gave phenotypes somewhat less severe than those given by *ptc*-GAL4-driven UAS-*Bar*, suggesting that *ptc*-GAL4 is a slightly stronger driver than *blk*-GAL4. Among 13 independent UAS-*Lim1* lines so far generated, nine lines, showing essentially the same phenotype when driven by *blk*-GAL4, were chosen and used for further experiments. UAS-*Lim1* was driven only by *blk*-GAL4, since flies with *ptc*-GAL4 and UAS-*Lim1* were mainly larval lethal under our experimental conditions.

**Immunohistochemistry and in situ hybridisation**

X-Gal staining and antibody staining were carried out according to Sato et al. (1999b). Primary antibodies used were rat anti-AL (Campbell et al., 1993), mouse anti-DLL (Diaz-Benjumea et al., 1994), rabbit anti-BarH1 (Higashijima et al., 1992b), mouse anti-FAS2 (Lin et al., 1994), rabbit anti-*lacZ* (anti- $\beta$ -galactosidase; Cappell), and mouse anti-*lacZ* (Promega). As secondary antibodies, Cy3, Cy5 (Amersham Pharmacia Biotech), or biotin (vector) conjugated antibodies followed by avidin-FITC (Promega) were used. Images were obtained using MRC-1000 confocal microscopy (Bio Rad) and processed using Photoshop 5.0 (Adobe). In situ hybridisation was carried out as described previously (Sato et al., 1999a). RNA probe was prepared using the GH04929 insert as a template.

***Xenopus* embryo injection assay**

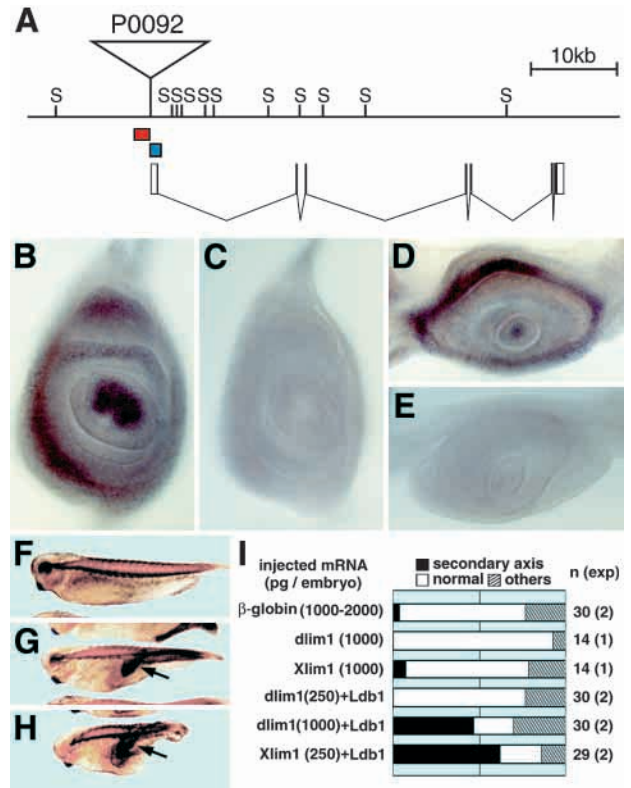
Plasmids for mRNA injections were constructed by inserting PCR amplified 1.5 kb *Lim1*-coding sequences between *Bam*HI and *Xba*I sites of pCS2+ (Turner and Weintraub, 1994). *Xlim1* constructs, mRNA synthesis, *Xenopus* embryo injections and antibody staining for 12/101 antibody (Kintner and Brockes, 1984) have been described previously (Taira et al., 1994).

**RESULTS**

**Identification of *Lim1* as a gene expressed in the distal tips of developing legs and antenna**

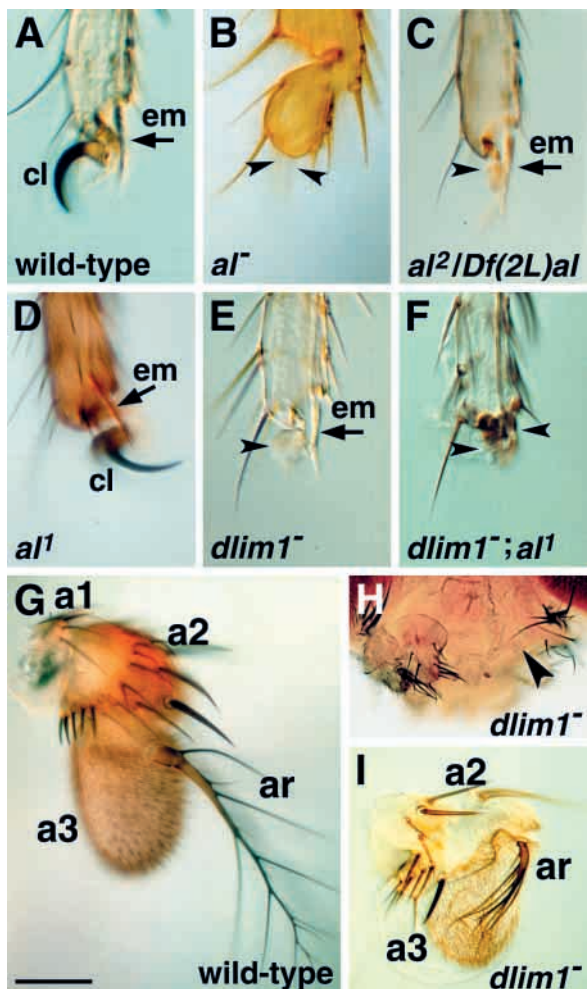
To isolate genes that possibly act with *al* in pretarsus specification, a search was made for genes expressed in the pretarsus but not the segment immediately adjacent to it at late third instar stages. P0092 is an enhancer trap line, in which *lacZ* expression in leg and antennal discs was found to be similar to *al* expression in these tissues. As shown in Fig. 1, *lacZ* was coexpressed in virtually all AL-positive cells in the pretarsus, tibia, femur and possibly coxa in leg discs (Fig. 1B-D), and the arista and first antennal segment in antennal discs (Fig. 1E-E'). Although AL expression was restricted to ventral cells in the tibia and dorsal cells in the femur, coxa and first antennal segment, *lacZ* expression was noted in both ventral and dorsal cells uniformly, which gave rise to complete circular expression. In wing and haltere discs, in which *al* is also expressed (Campbell et al., 1993), no appreciable expression of P0092-*lacZ* was observed (data not shown).

In P0092, P-element insertion occurs at 8B on the X chromosome (FlyView). Genomic DNA clones spanning about 70kb region surrounding the P insertion site were isolated



**Fig. 2.** (A) Genomic organisation of the *Lim1* (*dlim1*) locus. The triangle indicates the P insertion site in P0092. S shows *Sal*I sites. Boxes below the map represent coding and non-coding exons, respectively. Red box, a DNA fragment isolated by plasmid rescue. Blue box, a deletion found in *Lim1*<sup>7B2</sup>. This *Lim1*<sup>7B2</sup> deletion includes RNA start, the entire 5' exon and part of the first intron. (B-E) Expression of *Lim1* mRNA in wild-type leg (B), *Lim1*<sup>7B2</sup> leg (C), wild-type antenna (D) and *Lim1*<sup>7B2</sup> antenna (E) discs. Note that *Lim1* mRNA expression pattern is similar to that of *Lim1-lacZ* (see Fig. 1B-E'). (F-I) Functional assay of *Lim1* in *Xenopus* embryos. (F-H) Muscle patterns revealed by 12/101 antibody staining of embryos injected with *Lim1* (F), *Lim1*+*XLdb1* (G) and *Xlim1*+*XLdb1* (H) mRNAs. The final dose of mRNA per embryo was 1, 0.5 and 0.25 ng for *Lim1*, *Xenopus Ldb1* and *Xlim1*, respectively. Arrows show ectopic muscles generated through secondary axis formation. (I) Incidence of secondary axis formation. Embryos were injected with mRNA indicated and scored for axis development at the tailbud stage (stage 33/34) and categorised as secondary axis (black bars), normal (white bars), or others (hatched bars; embryos with reduced axis or incomplete blastopore closure, probably due to gastrulation defects). The numeric figures on the right indicate the number of total embryos (*n*) from one or two experiments as indicated in parentheses.

using a plasmid rescue fragment as an initial probe (Fig. 2A). Two relevant EST (Expressed Sequence Tag) clones (GH04929 and LD27231) were identified in the Berkeley Drosophila Genome project (BDGP) database using genomic DNA sequence information. As shown in Fig. 2B,D, GH04929 gave in situ hybridisation patterns almost identical to P0092-*lacZ* expression. Nucleotide sequence analysis indicated that the putative P0092 gene encodes a LIM-homeodomain protein identical in amino acid sequence to LIM1, a *Drosophila* homologue of vertebrate LIM1 (Accession number, AB034690; Lilly et al., 1999).



**Fig. 3.** Pretarsus and antennal phenotypes of *Lim1* (*dlim1*) null mutants. Arrows indicate empodia, whereas arrowheads indicate the absence of wild-type structures. (A-F) Pretarsus structures of wild-type (A), *Lim1*<sup>7B2</sup> (E), *al*<sup>2</sup>/*Df(2L)al* (C), *al*<sup>-</sup> (*al*<sup>30</sup>/*alice*, *al*<sup>1</sup>/*Df(2L)al*) (D) and *Lim1*<sup>-</sup>; *al*<sup>1</sup> (F) legs. Proximal is towards the top. Note that *Lim1*<sup>-</sup> mutants claws (cl) are completely abolished while both claws and empodium (em) are absent from *Lim1*<sup>-</sup>; *al*<sup>1</sup> mutants. (G-I) Antenna of a wild-type (G) and *Lim1*<sup>-</sup> (H, I) flies. ar, arista. a1-a3, the first-third antennal segments, respectively. In *Lim1*<sup>-</sup> flies, entire antennal structures were lost in 50% of times (see the arrowhead in (H)). (I) shows that *Lim1*<sup>-</sup> mutant antenna lack the first antennal segment and possess arista that are severely deformed. Scale bar: 30  $\mu$ m in A-F; 80  $\mu$ m in H; 60  $\mu$ m in G, I.

*Xlim1* is known to initiate the formation of a secondary axis when its mRNA is coinjected with *XLdb1* mRNA, which encodes a LIM domain-binding protein homologous in amino acid sequence to *Drosophila* Chip (Agulnick et al., 1996; Morcillo et al., 1997). Thus, a study was undertaken to determine whether *Lim1* possesses activity similar to *Xlim1* by injecting *Lim1* mRNA into fertilised *Xenopus* eggs with *XLdb1* mRNA. Figure 2F-I show that, as with *Xlim1*, *Lim1* is capable of effectively inducing a secondary axis in *Xenopus* in a *XLdb1*-dependent manner. It may thus follow that the P0092 gene product or *Drosophila* LIM1 is similar not only in amino acid sequence but also in association with LIM domain-binding protein and target sequence recognition to vertebrate LIM1.

### *Lim1* is essential for leg and antennal distal structure formation

Flies neither homozygous nor hemizygous for the P0092 P insertion showed any obvious morphological defects. Thus, *Lim1* loss-of-function mutants were generated by imprecise P-element excision and six independent larval or pupal lethal mutant lines were obtained. These frequently produced pharate adults with apparent defects in mouth parts, leg and antennal morphology (for detailed mutant phenotypes, see below), making it possible to examine the roles of *Lim1* in leg and antennal development.

*Lim1*<sup>7B2</sup> was the severest in our *Lim1* mutants. In this mutant, the predicted RNA start site, the first exon and a portion of the first intron were found to be lost (Fig. 2A). No appreciable *Lim1* RNA signals could be detected in *Lim1*<sup>7B2</sup> leg and antennal discs (Fig. 2C,E) and embryos (not shown), indicating that it is a transcriptional null mutant allele. In the following, *Lim1*<sup>7B2</sup> is referred to as *Lim1*<sup>-</sup> and used as the *Lim1* mutant.

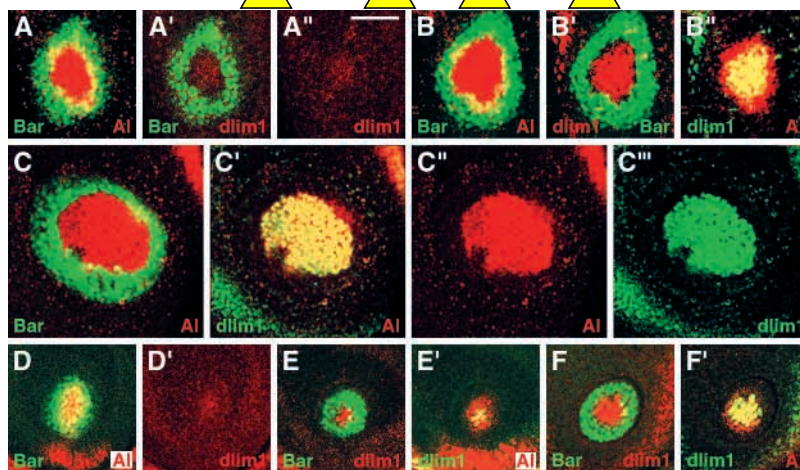
In legs and antenna completely lacking *al* activity, all pretarsus structures and arista are lost, respectively (Fig. 3B; Campbell and Tomlinson, 1998). In moderate hypomorphic *al* mutants such as *al*<sup>130</sup>, *al*<sup>1</sup>/*Df(2L)al* and *al*<sup>2</sup>/*Df(2L)al* flies, claws are frequently lost without loss of other pretarsus structures such as pulvilli and empodia (Fig. 3C; Schneitz et al., 1993; Campbell and Tomlinson, 1998; Kojima et al., 2000), while in weak hypomorphic mutants (e.g. homozygotes for *al*<sup>1</sup>), claws and arista were not lost but only reduced in size (Fig. 3D). Figure 3E shows that, in *Lim1*<sup>-</sup> legs, pulvilli and empodia were normally present but claws are frequently lost. It may thus follow that *Lim1*<sup>-</sup> mutants are very similar in leg phenotype to moderate *al* hypomorphic mutants. In about half of all cases ( $n=24$ ), the antenna was absent from the *Lim1*<sup>-</sup> half head (Fig. 3H). When antennae were present, arista was deformed and reduced in size (Fig. 3I). That is, *dlim1*<sup>-</sup> arista are morphologically similar to those of weak hypomorphic *al* mutants. These findings indicate that *Lim1* is essential for proper development of pretarsus and arista as well as *al*, although *Lim1*<sup>-</sup> mutant phenotypes are much less severe than *al*<sup>-</sup> mutant phenotypes. In *Lim1*<sup>-</sup> legs that were simultaneously homozygous for *al*<sup>1</sup>, not only claws but also empodia and pulvilli were frequently lost (Fig. 3F). The concerted function of *Lim1* and *al* would thus appear to be required for normal pretarsus/arista development.

### Absence of *Lim1* expression in early pretarsus and arista precursor cells expressing *al*

*Lim1* may affect the morphogenesis of distal parts of the leg and antenna by modulating *al* and/or *Bar* expression or their mutually antagonistic interactions (Kojima et al., 2000). *Lim1* expression may be affected by *al* and/or *Bar* activity. As a first step to clarify these points, examination was made of *Lim1-lacZ*, *al* and *Bar* expression in the centre of developing wild-type leg and antennal discs. Gene expression was examined using the corresponding antibodies for the gene products.

As indicated previously, AL and BAR expression begins in the central region of early-third instar leg discs in a partially overlapping manner (Fig. 4A; Kojima et al., 2000). As shown in Fig. 4A', no *Lim1-lacZ* expression was noted to occur at the earliest stages of AL and BAR expression in early third instar. Just prior to initiation of central fold formation along the outer

**Fig. 4.** Expression patterns of *Lim1* (*dlim1*), *al* and *Bar* in distal tips of leg (A-C'') and antennal (D-F') discs. Leg and antennal discs were stained simultaneously for *Lim1-lacZ*, AL and BAR. Signals and corresponding colours are indicated by coloured letters in each figure. (A-A'') At the onset of the expression of AL (A, red) and BAR (A.A', green) at early third instar: little, if any, *Lim1-lacZ* expression (A',A'', red) was observed. Note that overlapping between AL and BAR expression is seen as yellow signals. (B-B'') Just prior to the initiation of central folding along the outer circumference of the BAR ring (B.B', green), *Lim1-lacZ* started to be expressed (B', red; B'', green). Note that the *Lim1-lacZ* domain (B'', green) is smaller than and included within the AL domain (B'', red), and that there is little or no overlap between *Lim1-lacZ* (B', red) and BAR (B', green) expression. (C-C'') Staining patterns at mid third instar. As described previously (Kojima et al., 2000), there is no overlap between BAR (C, green) and AL (C, red) expression domains. Note that *Lim1-lacZ* (C',C'': green) and AL (C',C'': red) expression domains are almost identical in size and shape to each other. (D-F') As in the case of leg discs, *Lim1-lacZ* in antennal discs began to be expressed after the onset of AL and BAR expression (D-E'): initially, *Lim1-lacZ* expression occurred within the AL expression domain (E,E'). At late third instar, *Lim1* expression expanded without overlapping with the BAR ring (F). Scale bar: 50  $\mu$ m.



circumference of the BAR ring (Kojima et al., 2000), *Lim1-lacZ* expression first became detectable in the centre of the AL domain (Fig. 4B-B''). But, unlike the AL domain, the *Lim1-lacZ* expression domain did not overlap the surrounding BAR expression domain (Fig. 4B'). By mid third instar, the central region of the leg disc had divided almost completely into two non-overlapping regions; the central domain expressing AL and *Lim1-lacZ* but not BAR, and the surrounding domain expressing only BAR (Fig. 4C-C''). A similar relationship between AL, BAR and *Lim1-lacZ* expression was observed in antennal discs (Fig. 4D-F').

### Requirements of *al* for *Lim1* expression and *Bar* repression in the pretarsus

That *Lim1-lacZ* expression is initiated in the AL domain would suggest that *al* is required for *Lim1* expression. Thus, we examined whether *Lim1-lacZ* expression would be affected by misexpressing *al* along the A/P border using *ptc*-GAL4 and UAS-*al* or by abolishing *al* activity from the presumptive pretarsus. Although no *Lim1-lacZ* misexpression was induced by ectopic *al* expression along the A/P border (Fig. 5A-B), pretarsus *Lim1-lacZ* expression was virtually completely eliminated in *al*<sup>-</sup> leg discs (Fig. 5C'), indicating that *al* is directly or indirectly required for pretarsus *Lim1* expression.

Since partial *Bar* misexpression was previously observed in *al* hypomorphic mutants (Kojima et al., 2000), *Bar* misexpression might be induced throughout the presumptive pretarsus in *al*<sup>-</sup> leg discs. We tested this hypothesis and found that this is the case. As shown in Fig. 5C, *Bar* was misexpressed strongly over the entire presumptive pretarsus region of *al*<sup>-</sup> leg discs. Loss of *Lim1* expression in *al*<sup>-</sup> leg discs may thus arise from a secondary effect of *Bar* misexpression in the absence of *al* activity.

### Repression of *Lim1* expression by *Bar* misexpression

To clarify whether *Bar* is capable of repressing the pretarsus *Lim1* expression, UAS-*BarH1* was driven by *ptc*-GAL4 to determine the effects of *Bar* misexpression on the pretarsus

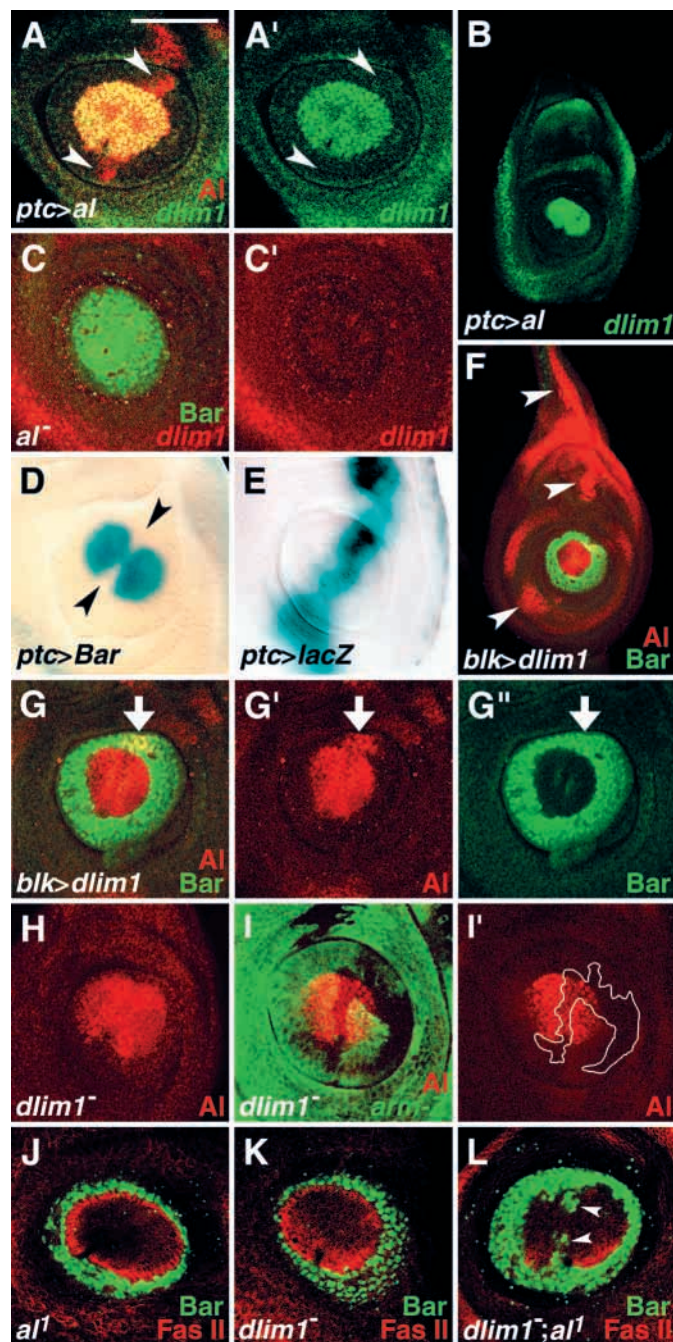
*Lim1-lacZ* expression. As shown in Fig. 5D,E, endogenous *Lim1-lacZ* expression was almost completely abolished along the A/P border where *Bar* was misexpressed, thus confirming that *Bar* is capable of repressing *Lim1* expression in the presumptive pretarsus. Hence, the idea that loss of *Lim1* in *al*<sup>-</sup> leg discs is caused by *Bar* misexpression, which is induced in the absence of *al* activity, was again supported, although the possibility that *al* activates *Lim1* expression independently of *Bar* cannot be formally excluded.

### Requirement of *Lim1* for *al* expression

*Lim1* expression requires *al* activity but this does not necessarily rule out the possibility that *al* expression is governed by *Lim1*. To confirm this, *Lim1* was misexpressed along the A/P border using *blk*-GAL4 and UAS-*Lim1* (see Materials and Methods) or mosaic clones mutant for *Lim1* were made. Fig. 5F-G'' demonstrate that AL misexpression results from *Lim1* misexpression not only in the BAR domain but more proximal regions as well. In contrast, although AL expression in the pretarsus was not completely eliminated in *Lim1*<sup>-</sup> leg discs (Fig. 5H), it was evident that AL expression was significantly reduced in a cell-autonomous fashion in *Lim1*<sup>-</sup> clones generated in the pretarsus (Fig. 5I,J). Moreover, AL expression in the region other than the pretarsus was also substantially reduced or completely eliminated, as described below (see Fig. 6). Therefore, *Lim1* probably activates *al* expression in all *al*-expressing leg and antennal cells, including those in the pretarsus.

### Involvement of *Lim1* and *al* in normal smooth border formation between the pretarsus and tarsal segment 5

Formation of the tarsal segment 5/pretarsus boundary requires antagonistic interactions between *Bar* and *al* (Kojima et al., 2000). To determine whether *Lim1* is involved in this process, the effects of the absence of *Lim1* activity on *Bar* expression were examined. As with *al* hypomorphic mutants, BAR expression appeared virtually normal in nearly all cases (Fig. 5J,K, Table 1). However, about 80% of leg discs showed BAR



misexpression in the pretarsus in double mutants of *Lim1*<sup>-</sup> and *al*<sup>1</sup> (Fig. 5L, Table 1), indicating the involvement of *Lim1* in the repression of *Bar* expression.

Fasciclin 2 (FAS2), a putative  $\Delta$  protein involved in cell-cell connection (Grenningloh et al., 1991), is strongly expressed in border cells separating the pretarsus and tarsal segment 5 cells (Kojima et al., 2000). Although FAS2 expression was almost normal in *al*<sup>1</sup> discs (Fig. 5J) and only slightly reduced in *Lim1*<sup>-</sup> discs (Fig. 5K), most FAS2 expression was eliminated in double mutants (Fig. 5L), indicating that both *al* and *Lim1* are involved in the regulation of FAS2 expression in border cells. Interestingly, the normal smooth boundary between the pretarsus and tarsal segment 5 was replaced by irregularly

**Fig. 5.** Interactions between *Lim1* (*dlim1*), *al* and *Bar* in the distal region of the leg disc. (A-B) Expression of AL (red) and *Lim1-lacZ* (green) in *ptc-GAL4/UAS-al*<sup>6</sup> leg discs. Arrowheads indicate ectopic AL expression along the A/P border. In (A',B), only *Lim1-lacZ* expression is shown. No induction of *Lim1-lacZ* misexpression was observed in the entire leg disc upon *al* misexpression. (C,C') BAR (C, green) and *Lim1-lacZ* (C', red) expression in an *al*<sup>-</sup> (*al*<sup>ex/al</sup><sup>ice</sup>) leg disc. BAR was misexpressed over the entire presumptive pretarsus, while pretarsus *Lim1-lacZ* expression was completely eliminated. (D,E) X-Gal staining of *Lim1-lacZ/UAS-BarH1*<sup>M6</sup>, *ptc-GAL4/+* (D) or *ptc-GAL4/+; UAS-lacZ/+* (E) leg discs. Arrowheads in D show the repression of *Lim1-lacZ* expression by *Bar* misexpression. (F-G'') BAR (green) and AL (red) expression in *UAS-dlim1*<sup>111F/+</sup>; *blk-GAL4/+* leg discs, where *Lim1* is misexpressed along the A/P border. Arrowheads in F indicate AL misexpression while arrows in (G-G'') indicate that AL misexpression but no BAR repression occur in the BAR domain. (H-I') The pretarsus AL expression (red) in a *Lim1*<sup>-</sup> (*Lim1*<sup>7B2</sup>) mutant leg disc (H) or a *Lim1*<sup>-</sup> mosaic clone (I,I'). The clone is indicated by the absence of *arm-lacZ* (green; I) or outlined (I'). Note that AL signals are considerably reduced in the *Lim1*<sup>-</sup> clone. (J-L), BAR (green) and FAS2 (red) expression in *al*<sup>1</sup> (J), *Lim1*<sup>-</sup> (K) and *Lim1*<sup>-</sup>; *al*<sup>1</sup> mutant leg discs. In the double mutant, FAS2 expression was extensively reduced and patchy BAR misexpression was frequently observed (arrowheads in L). All discs are from late third instar larvae. Dorsal is towards the top and anterior towards the left. Scale bar: 50  $\mu$ m in A,A',C-E,G-L; 100  $\mu$ m in B,F.

**Table 1. Genetic interactions between *al*, *Lim1* and *Bar***

Genotypes	<i>Bar</i> misexpression*	<i>n</i> †
+ <i>Y</i> ; <i>al</i> <sup>1</sup> / <i>al</i> <sup>1</sup>	2 (4%)	45
<i>Lim1</i> <sup>7B2</sup> / <i>Y</i> ; +/+	2 (4%)	56
<i>Lim1</i> <sup>7B2</sup> / <i>Y</i> ; <i>al</i> <sup>1</sup> / <i>al</i> <sup>1</sup>	33 (79%)	42

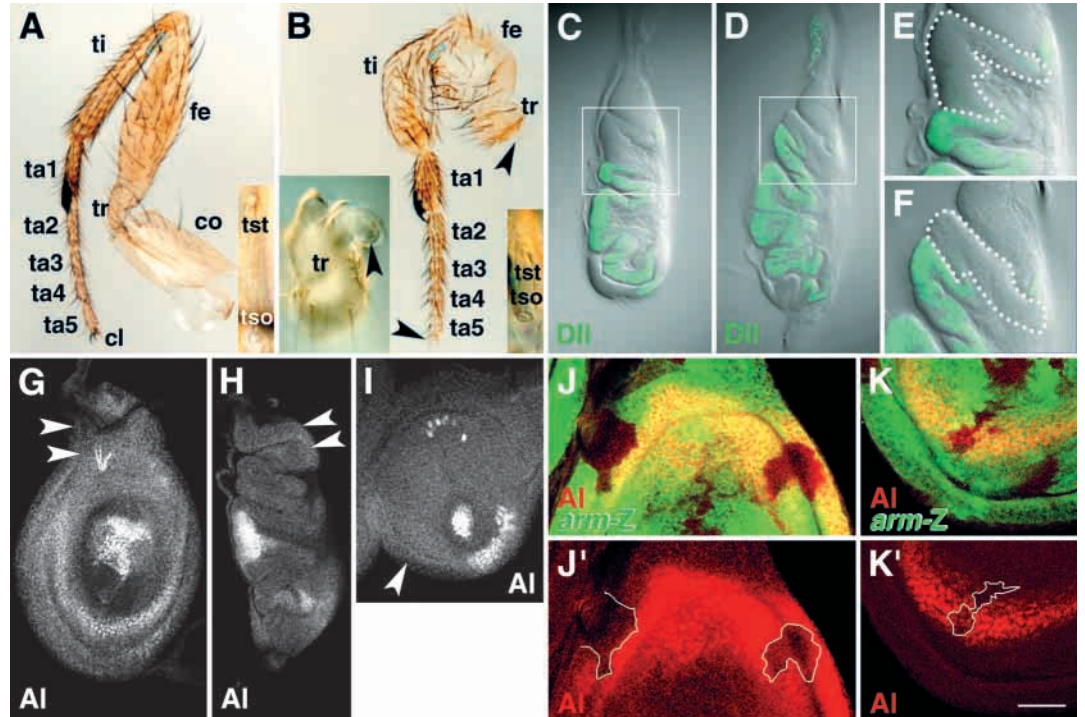
\*Number of leg discs showing *Bar* misexpression in the pretarsus. The percentage of total number of leg discs examined is shown in parentheses.  
 †Number of leg discs examined.

zigzagged one in the double mutant discs (Fig. 5L). However, it should be noted that any appreciable change in morphology of the pretarsus/tarsus boundary cannot be brought about solely by eliminating FAS2 activity (data not shown), suggesting the involvement of unknown factors functionally redundant to FAS2 in normal pretarsus/tarsus boundary formation.

### Requirement of *Lim1* for normal development of the femur and coxa

Apart from the future pretarsus, *Lim1* was expressed circularly in proximal segments such as the coxa, femur and tibia (see Fig. 1). In *Lim1*<sup>-</sup> flies, the femur was extensively reduced in size (Fig. 6A,B) and the coxa was missing for the most part or present only as a small bulb-like structure (Fig. 6B, left inset), suggesting the requirement of *Lim1* for proper development of the femur and coxa. Although the tibia was bent and fused with the femur, morphological analysis indicated the presence of essentially normal characteristic structures of the tibia, such as transverse rows of bristles, preapical bristles, tibial sense organs and tibial sensilla trichodea (Bryant, 1978; data not shown); tibial sense organs and tibial sensilla trichodea are structures situated near the proximal tibial end (Fig. 6A,B, right inset). The tibial phenotype may thus possibly derive from secondary effects of the femoral deformation. In late third

**Fig. 6.** Requirements of *Lim1* (*dlim1*) for the formation of proximal leg segments. (A,B) Prothoracic legs of wild-type (A) or *Lim1*<sup>-</sup> (*Lim1*<sup>7B2</sup>; B) male flies. Note that extreme shortening of the femur (fe) and the absence of the coxa (co) and claws (cl) (arrowheads) in (B). Left inset in B shows an enlargement of the proximal end of a mutant leg. The arrowhead indicates a rudimentary segment possibly corresponding to the coxa. Right insets in A,B are enlargements of the boxed regions near the proximal end of the tibia (ti), showing tibial sense organs (tso) and tibial sensilla trichodea (tst). ta1-ta5, tarsal segments 1-5; tr, trochanter. (C-F) Sagittal views of wild-type (C,E) or *Lim1*<sup>-</sup> (D,F) discs at late third instar stained for DLL (green). Signals are combined with Nomarsky images. (E,F) Magnified views of boxed regions in (C,D), respectively. The region flanked by two DLL domains (enclosed by broken lines) are much narrower in a *Lim1*<sup>-</sup> disc than in a wild-type disc. (G-I) AL expression in late third instar leg discs (G,H) and an antennal disc (I) of *Lim1*<sup>-</sup> flies. (H) is a sagittal optical section. Arrowheads indicate the loss of wild-type AL expression (see Fig. 1B",C",E"). (J-K') Absence or reduction of AL expression (red) in *Lim1*<sup>-</sup> mosaic clones in the femur/coxa (J,J') or tibia (K,K'). Clones are marked by the absence of *arm-lacZ* (green, J,K) or outlined (J',K'). In all figures except for A,B, dorsal is towards the top. Scale bar: 110 μm in A,B; 50 μm in C,D,G-I; 25 μm in E,F,J-K'.



instar, DLL expression is evident in the central region spanning from the most distal tip to distal half of the tibia along with in the future trochanter (Fig. 6C, see also Fig. 1A; Diaz-Benjumea et al., 1994). Consistent with shortening of the femur, appreciable reduction in mass has already taken place in the region flanked by the central DLL domain and the proximal DLL ring at late third instar (Fig. 6C-F).

In *Lim1*<sup>-</sup> leg and antennal discs, AL expression in the proximal region, such as in the femur, coxa and first antennal segment, was virtually absent (Fig. 6G-I). In *Lim1* mosaic clones in the femur or coxa, AL expression was abolished cell autonomously (Fig. 6J,J'). Tibial AL expression remained in *Lim1* discs (Fig. 6G,H) mosaic analysis clearly indicated substantial reduction in AL expression in *Lim1* clones (Fig. 6K,K'). But loss of AL expression would not completely explain the femoral and coxal defects, since *al* is dispensable for normal development of the femur and coxa (Campbell and Tomlinson, 1998).

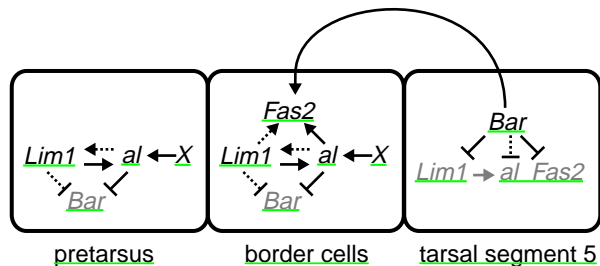
## DISCUSSION

### Possible roles of *Lim1* in pretarsus development

We showed here that *Lim1* is coexpressed with *al* in the future pretarsus (Figs 1 and 4) and required for proper pretarsus development (Fig. 3E). Since the pretarsus phenotype of *Lim1*<sup>-</sup> legs was similar to that of moderate *al* hypomorphic mutant legs (see Fig. 3C,E), the requirement of *Lim1* for pretarsus

formation may be less than that of *al*. The pretarsus phenotype of *Lim1*<sup>-</sup> legs was enhanced in double mutants of *al*<sup>ll</sup> (a very weak hypomorphic *al* allele) and *Lim1*<sup>-</sup> (Fig. 3F) indicating that *Lim1* and *al* are cooperatively involved in pretarsus development.

According to this, and the fact that *Lim1* expression in the future pretarsus is completely eliminated in *al*<sup>-</sup> leg discs (Fig. 5C'), *Lim1* might be considered to lie downstream of *al* and be involved in only some *al* functions. However, normal levels of pretarsus AL expression required *Lim1* activity (Fig. 5I,I') and *Lim1* misexpression induced AL misexpression (Fig. 5K,K'), indicating that LIM1 rather serves as an activator of *al* expression. Furthermore, *Bar* misexpression in the pretarsus caused repression of *Lim1-lacZ* expression (Fig. 5D) while *al* misexpression failed to induce ectopic *Lim1-lacZ* expression (Fig. 5A,B), implying that the elimination of pretarsus *Lim1-lacZ* expression in *al*<sup>-</sup> leg discs is an indirect consequence of the absence of *al* activity through strong *Bar* misexpression (Fig. 5C). All these findings and considerations are consistent with the idea that *Lim1* lies upstream of *al* and at least some *Lim1* functions in the pretarsus are mediated by activation of *al* expression (see a solid arrow in Fig. 7), although the possibility that the pretarsus *Lim1* expression is partly under the direct positive control of *al* cannot be formally excluded (see a broken arrow in Fig. 7). *al* is expressed considerably prior to that of *Lim1* (Fig. 4A-A') and *Lim1* may thus be involved in maintenance of pretarsus *al* expression. The incomplete elimination of pretarsus *al* expression in *Lim1*<sup>-</sup>



**Fig. 7.** The most likely regulatory relationships between *al*, *Lim1* and *Bar* are shown by unbroken lines. Black, expressed genes; grey, repressed genes. Broken lines indicate alternative but not exclusive pathways of gene interactions. See the text for details.

discs (see Figs 5H-I' and 6G,H,K,K') indicates the involvement of one or more positive factors (designated as X in Fig. 7) other than *Lim1* in pretarsus *al* expression.

Mutually antagonistic interactions between *al* and *Bar* were previously shown to be essential for the strict separation of AL and BAR domains, leading to localized *Fas2* induction by *Bar* in border cells (Kojima et al., 2000). Although the absence of *Lim1* shows little BAR misexpression in the pretarsus (Fig. 5K, Table 1), increased BAR misexpression in *Lim1*<sup>-</sup>; *al*<sup>l</sup> leg discs (Fig. 5L, Table 1) could indicate the involvement of *Lim1* in the repression of *Bar* expression in the pretarsus (Fig. 7). Remarkable decrease in FAS2 expression in putative *Lim1*<sup>-</sup>; *al*<sup>l</sup> mutant border cells (Fig. 5L) indicates that *Fas2* expression requires *al* and *Lim1* functions, in addition to cell non-autonomous functions of *Bar* (Kojima et al., 2000; Fig. 7). *Lim1* may be involved in pretarsus specification and boundary formation only through its activation of *al*, as shown by the unbroken lines in Fig. 7. Low *al* expression in *Lim1* single mutants may still be sufficient for maintaining the normal expression of *Bar* and *Fas2*, but with further reduction in *al* expression in *Lim1*<sup>-</sup>; *al*<sup>l</sup> double mutants, *Bar* misexpression and loss of *Fas2* expression may result. Alternatively, as shown by broken lines in Fig. 7, *Lim1* may act independently of *al*, and simultaneous reduction in *al* and *Lim1* expression may cause *Bar* misexpression and reduction of *Fas2* expression in the double mutants. These considerations are not mutually exclusive.

Previous experiments have shown that pretarsus *al* expression is partially repressed by misexpressed *Bar* (Kojima et al., 2000). UAS-*Bar* driven by *ptc*-GAL4 repressed pretarsus *Lim1* expression along the A/P border almost completely (see Fig. 5D), while *al* expression was abolished only partially in *Lim1* mutants. Thus, *Bar* is likely to repress *al* expression indirectly through the repression of *Lim1* expression as depicted by unbroken lines in Fig. 7, although direct repression of *al* expression by *Bar* cannot be formally excluded. Taken together, our results indicate that the interactions between *al*, *Lim1* and *Bar* are important for precisely defining the pretarsus region.

*Lim1* misexpression indicated no appreciable reduction in *Bar* expression, even though AL misexpression was induced in the BAR domain (Fig. 5G-G'). *al* and *Lim1* may thus not be sufficient for repressing *Bar* expression in the future pretarsus. Interestingly, an additional locus ('*clawless*' locus) showing pretarsus defects similar to *al* mutants when mutated was recently found (T. K. and K. S., unpublished). In *clawless*

mutant leg discs, *Bar* is misexpressed in the presumptive pretarsus region as in the case of *al* mutants.

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