Chip-mediated partnerships of the homeodomain proteins Bar and Aristaless with the LIM-HOM proteins Apterous and Lim1 regulate distal leg development

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Summary

Proximodistal patterning in *Drosophila* requires division of the developing leg into increasingly smaller, discrete domains of gene function. The LIM-HOM transcription factors *apterous* (*ap*) and *Lim1* (also known as *dlim1*), and the homeobox genes *Bar* and *aristaless* (*al*) are part of the gene battery required for the development of specific leg segments. Our genetic results show that there are posttranslational interactions between Ap, Bar and the LIM-domain binding protein Chip in tarsus four, and between Al, Lim1 and Chip in the pretarsus, and that these interactions depend on the presence of balanced amounts of such proteins. We also observe in vitro protein binding between Bar and Chip, Bar and Ap, Lim1 and Chip, and Al and Chip. Together with the previous evidence for interactions between Ap and Chip, these results suggest that these transcription factors form protein complexes during leg development. We propose that the different developmental outcomes of LIM-HOM function are due to the precise identity and dosage of the interacting partners present in a given cell.

Key words: LIM-HOM, Prd-HOM, Chip, Apterous, Legs, *Drosophila*, DLim1

Introduction

Homeodomain (HOM) proteins play fundamental roles in development. The common feature that characterises this protein family is the presence of a homeodomain, which is involved mainly in DNA interactions (Gehring et al., 1994). Assays of DNA-binding specificity with homeodomains of different HOM proteins have shown that they bind to the same core target sequence. However, HOM proteins regulate specific sets of downstream genes in vivo, suggesting that other factors contribute to their specificity. The members of the LIM-HOM subfamily are characterised by the presence of two tandem repeated LIM domains in the N-terminal end of the protein followed by a homeodomain. Each LIM domain consists of two tandem cystine-histidine-rich zinc-fingers (Curtiss and Heilig, 1998; Jurata and Gill, 1998). The LIM domains do not seem to bind DNA, instead they are implicated in specific protein-protein interactions (Arber and Caroni, 1996; Schmeichel and Beckerle, 1994). LIM-HOM proteins are involved in a wide variety of developmental processes, and it has been suggested that LIM-HOM proteins regulate specific genes by forming multiprotein transcriptional complexes with other LIM-HOM proteins and/or other cofactors (Dawid et al., 1998; Hobert and Westphal, 2000). The LIM-domain binding proteins (Ldb) can bind LIM domains with high affinity and have been found in mouse (Ldb1/Nl1/Clim2), Zebrafish (Ldb4), *Xenopus* (Xldb1) and *Drosophila* (Chip). Ldb proteins also contain a homodimerisation domain, and can act as a bridge dimer between two LIM proteins to form homo- and hetero-tetrameric complexes with LIM-HOM transcription factors (Jurata et al., 1998).

Experiments in *Drosophila* have identified interactions in vivo between Chip and the LIM-HOM protein Apterous (Ap). Apterous is required for dorsoventral (DV) patterning and growth of the wing (Cohen et al., 1992). Dosage interactions and other genetic experiments involving *Chip* and *ap*, plus biochemical assays, have indicated that Ap function is carried out by a tetramer complex comprising two molecules of Ap bridged by a Chip dimer (Fernández-Fúnez et al., 1998; Milan and Cohen, 1999; Morcillo et al., 1997; Rincon-Limas et al., 2000; van Meyel et al., 1999).

Ap function in the wing is regulated by Dlmo (Bx – FlyBase; the *Drosophila* homologue of LIM-only, a protein composed only of LIM domains), which interacts with Chip with higher affinity than Ap to reduce the formation of active Ap-Chip complexes (Milan and Cohen, 2000; Shoresh et al., 1998; Weihe et al., 2001; Zeng et al., 1998). In the *Drosophila* CNS, Ap and Chip also interact physically and form tetrameric complexes required for the proper fasciculation of the *ap*-expressing interneurones. However, Ap function is regulated differently in the CNS than in the wing. For instance, *dlmo* (*Bx*) is not expressed in Ap neurones and the relative dosage between Ap and Chip is not limiting for the formation of Ap-Chip complexes (van Meyel et al., 2000). Furthermore, a combinatorial code between the LIM-HOM genes *islet* (*isl*; *tailup*, *tup* – FlyBase) and *Lim3* controls motoneurone pathway selection in flies and vertebrates (Thaler et al., 2002; Thor et al., 1999). In vertebrates, the combinatorial activities of Islet

and Lim3 homologues are not carried out by homo- or hetero-tetrameric complexes, instead they are carried out by a single Ldb-mediated hexameric complex (Thaler et al., 2002). Finally, it has been shown that Chip plays a role in other patterning processes by binding non-LIM proteins, such as the HOM proteins Bcd and Fz, and the GATA factor Pannier (Ramain et al., 2000; Torigoi et al., 2000). Therefore, Lbd specificity depends on the presence of different cofactors in each developmental context.

In the leg of *Drosophila*, a regulatory network of LIM-HOM and Prd-HOM (Paired-homeodomain) genes exists (Pueyo et al., 2000). The legs of *Drosophila* are formed from groups of epithelial cells, which segregate inside the embryo and grow during larval development, giving rise to sac-like structures called imaginal discs. The most distal part of the leg consists of five tarsal segments plus a pretarsus (Fig. 1A). Distal leg patterning first entails the establishment of the tarsal and pretarsal primordia at 80-90 hours after egg laying (AEL) (Galindo et al., 2002), followed by the subdivision of the tarsal field into smaller domains of gene expression (Fig. 1B-B‴) (Galindo and Couso, 2000). These domains define each tarsal segment (Kojima et al., 2000; Pueyo et al., 2000) and a joint is then intercalated between every segment (Bishop et al., 1999; de Celis et al., 1998; Rauskolb, 2001; Rauskolb and Irvine, 1999). Thus at 80-90 hours AEL the presumptive distal region of the leg disc appears divided into two domains of Prd-HOM gene expression: *aristaless* (*al*) expression in the pretarsus and *Bar* expression in the adjacent tarsal cells (Kojima et al., 2000). These patterns are activated by Distal-less (Dll) and a distal gradient of Egfr-Ras signalling (Campbell, 2002; Galindo et al., 2002). From 90 hours AEL, *Bar* expression is maintained at high levels by self-activation in the presumptive fifth tarsal segment, whereas in the fourth tarsal segment lower levels of Bar are required for the expression of *ap* (Fig. 1B-B‴). Consequently, a high dose of Bar protein and a low dose of Bar plus Ap are necessary for the development of the fifth and fourth tarsal segments, respectively. In the pretarsus, Al activates the expression of the LIM-HOM gene *Lim1* (also known as *dlim1*) after 90 hours AEL, and a positive-feedback mechanism and cooperation between them ensures pretarsal development. During this process, mutual repression between *Bar* on the one hand and *al* plus *Lim1* on the other establishes a sharp tarsal/pretarsal boundary (Pueyo et al., 2000; Tsuji et al., 2000).

In this study, we present more evidence for the existence of such a regulatory network, and suggest a role for direct protein

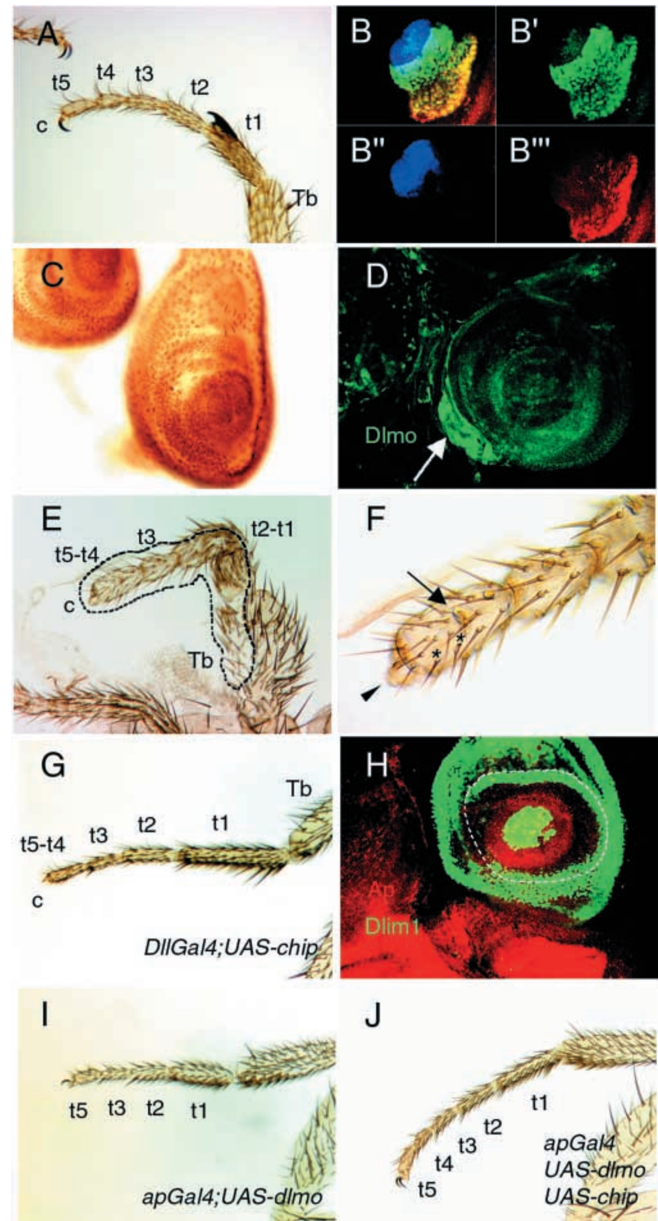

Fig. 1. The role of *Chip* and *dlmo* in leg development, and their genetic relationships with *apterous*. (A) The distal region of a wild-type prothoracic leg showing the distal part of the tibia (Tb), tarsal segments one to five (t1-t5), and the distalmost organ, the claw, in the pretarsus (c). (B-B‴) Each segment of the distal part of the leg is characterised by differential expression of the LIM-HOM Ap and Lim1 and the Prd-HOM Bar transcription factors. The images show a side view of an everting leg imaginal disc. B shows the merged triple staining; B′-B‴ show the separate channels. Expression of a *Bar* reporter gene in tarsus four and five is shown in green (B′); Lim1 protein distribution in the pretarsus is shown in blue (B″); and Ap protein distribution is shown in red (B‴; yellow in overlap in B). (C) Wild-type leg imaginal disc showing Chip protein distributed ubiquitously in the disc epithelium. (D) Dlmo protein distribution in a late third instar leg imaginal disc. Specific staining can be detected in a few cells in the peripodial membrane (arrow). (E) *Minute+* $Chip^{e55}$ clones in leg. The tissue lacking *Chip* is marked by its *yellow* (*y*) phenotype and is outlined in black. Clones in the tibia, femur, coxa and pretarsus show a phenotype similar to strong *Lim1* mutants. The fourth tarsal segment fails to develop, as in strong *ap* mutants. (F) Higher magnification of the tip of the leg shown in E. The majority of the distal part of the leg is y^- apart from two bristles that are y^+ (asterisks). In the pretarsus no claws develop (arrowhead). In addition, only a remnant part of a joint is observed between the last tarsal segments (arrow). (G) Leg of a *DllGal4;UAS-Chip* fly. Only four tarsal segments develop and the claw organ is absent, similar to the phenotype of *Chip* lack of function, which is shown in E. (H) Ap (red) and Lim1 (green) protein expression are normal in a *DllGal4;UAS-Chip* leg disc. The white dotted line denotes the edge of the distal domain of expression of the *DllGal4* line. (I) Leg of an *apGal4;UAS-dlmo* fly (29°C). Although the LIM-only gene (*dlmo*) is not expressed in the leg imaginal disc, Dlmo overexpression produces loss of the fourth tarsal segment. (J) Co-expression of *UAS-Chip* in an *apGal4;UAS-dlmo* genetic background rescues the loss of the fourth tarsal segment.

interactions, in addition to transcriptional regulation, in its mechanism. Genetic interactions and ectopic expression experiments highlight dosage relationships between Ap and Bar, and between Al and Lim1, and posttranslational dominant-negative interactions between these and other LIM-HOM proteins. The interactions between Ap, Bar, Al and Lim1 involve Chip, which we show to be required for the development of the distal leg regions. We show that protein interactions between Bar, Ap and Chip exist, leading to the suggestion that Ap-Chip-Bar protein complexes are the functional transcriptional units that control tarsus four development. In the presumptive pretarsus, a similar relationship between Lim1, Al and Chip exists. Synergistic cooperation between Al and Lim1 is required to direct pretarsus development, and to repress *Bar* expression and function. Ectopic expression of other LIM-HOM proteins in the pretarsus disrupts this cooperation, which also depends on Chip and is sensitive to changes in the dosage of the proteins involved. We reveal the existence of protein interactions between Al and Chip, suggesting that the Al-Chip-Lim1 protein complexes are the functional transcriptional units in the pretarsus. Thus, our results suggest that, in the fly leg, just as in the vertebrate head organiser (Nakano et al., 2000), LIM-HOM gene function is implemented by transcriptional complexes involving LIM-HOM/Chip/Prd-HOM proteins. We propose that the different developmental outcomes of LIM-HOM protein function are due to the precise identity and dosage of the co-factors available locally.

Materials and methods

Fly strains and genetic manipulations

Several fly strains in this paper were described by Pueyo et al. (Pueyo et al., 2000). Other stocks were: *UAS-Bar* (Kojima et al., 2000); *UAS-dlmo* (Zeng et al., 1998); hdp^{R26} (Milan et al., 1998); *UAS-ap∆HD*, *UAS-ap∆LIM* and *UAS-Lim3-ap* (O'Keefe et al., 1998); *UAS-Chip∆LID*, *UAS-Chip∆DD* and *UAS-Chip-ap* (van Meyel et al., 1999); and al^{ex} (Campbell and Tomlinson, 1998). The Gal4/UAS system was used to express genes in specific patterns of expression. The Gal4 drivers employed were: *dppGal4*, *apGal4* and *DllGal4*. All flies and larvae were raised at 25°C unless specified in the text. Clones of null *Bar* and *Chip* alleles were generated by the FRT/FLP system. In the generation of Bar^{-} clones, larvae of the genotype $Df(1)B^{263\text{-}20}$ *FRT 18A/yw hsGFPw*$^{+}$*FRT18A; hsFLP122/+* were heatshocked at 37°C for 90 minutes at 24-48 hours AEL ,and then transferred to 25°C. Bar^{-} cells in the adult were marked by the loss of the *forked* gene, which is included in $Df(1)Bar^{263\text{-}20}$. For staining of imaginal discs, larvae (100-120 hours AEL) were heatshocked again at 37°C for 1 hour to induce GFP expression, left to recover for one hour and then dissected. In the generation of null *Chip* clones, animals of the genotype *y w FLP; FRTG13* $Chip^{e55}$*/FRT42B* y^{+} *M(2)* (Morcillo et al., 1997) were heatshocked at 37°C for 90 minutes at 24-48 hours AEL. For the generation of null *Bar* clones, labelled as above, but expressing the *ap* transgene, males *FRT18A Ubi-GFP; DllGal4; UAS-FLP/+* were crossed to females $Df(1)B^{263\text{-}20}$ *FRT18A/FM7i; UAS-ap*. For the control cross, males *FRT18A Ubi-GFP; DllGal4; UAS-FLP/+* were crossed to females $Df(1)B^{263\text{-}20}$ *FRT18A/FM7i-GFP*. The progeny were raised at 18°C to avoid any mutant effects caused by the *DllGal4* driver. A similar experiment was performed using the weaker *babGal4* driver at 25°C, obtaining similar results.

GST pull-down assay

Glutathione-S-transferase (Gst)-Chip fusion constructs were generated and kindly provided by Dale Dorset (Torigoi et al., 2000). Gst-Ap and Gst-ApLim fusion constructs were generated by PCR amplification of the *ap* cDNA using specific primers. The same forward primer AGAGAGGATCCATGGGCGTCTGCACCGA was used in both amplifications, whereas the reverse primers were the Ap reverse primer GAGAGAGAATTCTTCCTGAGCATCCGTTAGTCC and the ApLim reverse primer GAGAGAGAATTCGCTATGCTG-TAGTGGGTC. PCR products were firstly cloned using TA cloning kit (Invitrogen). Positive clones were double digested with *Xho*I/*Eco*RI and the appropriate DNA fragment was gel extracted. Finally, the DNA fragment was cloned in the pGEX-2T vector (Amersham Pharmacia). Expression of the Gst-fusion proteins and binding to Gluthathione-agarose beads (Amersham Pharmacia) were performed as described by Torigoi et al. (Torigoi et al., 2000). Fly extracts were obtained by homogenisation of 50 brain and leg complexes from third instar larvae in dry ice, and resuspension in 150 µl of 50 mM Tris (pH 7.2), 150 mM NaCl, 2 mM EGTA, 5% Triton X-100 with protease inhibitors (Roche). 100 µl of blocked beads with the GST-fusion proteins were incubated with 300 µl of fly extract for 1 hour at 4°C. After the binding reaction, beads were washed three times with blocking solution, twice with PBT, and twice with PBS. 60 µl of 2×SDS reducing buffer/DTT were added to the beads and boiled. The samples were loaded in a 10% SDS-PAGE gel and analysed by western blot. Rabbit anti-BarH1 (Kojima et al., 2000) and guinea pig anti-Lim1 (Lilly et al., 1999) were used at a 1:5000 dilution, and Rat anti-Al (Campbell, 2002) was used at 1:10,000. Secondary antibodies coupled to peroxidase for rabbit and guinea pig were obtained from Dako and Jackson ImmunoResearch. Finally, the ECL system was used for detection of peroxidase reaction. In separate experiments, the TNT Quick Coupled Transcription/Translation Systems (Promega) were used to express *BarH1* cDNA.100 µl of beads with the GST-Chip fusion proteins were incubated with 100 µl of the TNT reaction. Following the washes the protocol was followed as above.

Immunocytochemistry

Antibody staining procedures were performed as described previously (Pueyo et al., 2000). Antibodies used were: guinea pig anti-Lim1 (Lilly et al., 1999); rat anti-Ap (Lundgren et al., 1995); rabbit anti-Dlmo (Milan et al., 1998); rabbit anti-β-galactosidase (Cappel). Secondary antibodies were obtained from Vector Laboratories and Jackson ImmunoResearch.

Results

The *apterous* and *Chip* genes interact during tarsus four development

The *ap* gene is expressed in the leg imaginal disc from 96 hours AEL in a ring of cells corresponding to the presumptive fourth tarsal segment (Fig. 1B,B‴) (Cohen et al., 1992). In strong *ap* mutants, the fourth tarsal segment is either absent or reduced in size, and is fused to the fifth tarsal segment (Fig. 2A) (Pueyo et al., 2000). This *ap* mutant phenotype is completely rescued by expression of a *UAS-ap* transgene (Fig. 2B), showing that Ap is required for the proper development of the fourth tarsal segment.

The *Chip* gene is expressed ubiquitously in the legs (Fig. 1C) (Morcillo et al., 1997) but no functional requirement has been reported. We have induced clones of cells lacking Chip and observe defects in different parts of the leg, such as tarsal segments four and five, the pretarsus, the tibia (Fig. 1E,F), and the coxa and femur (not shown). Given this requirement, and the functional relationship between Chip and Ap in the wing, we searched for possible genetic interactions between *ap* and *Chip*. First, transheterozygous allelic combinations between *ap* and *Chip* (*apGal4* or ap^{UGO35}/$Chip^{371}$, $Chip^{96.1}$, $Chip^{e55}$) did

not show any mutant phenotype in the legs (data not shown) (Pueyo et al., 2000). Second, *UAS-ap* and *UAS-Chip* full-length transgenes were overexpressed in the *ap* domain and in both cases no phenotype in the legs was observed (Pueyo et al., 2000), whereas the wings blister as described previously (Fernández-Fúnez et al., 1998). Finally, overexpression of a fragment of the Ap protein containing only the LIM domains, which interacts with Chip and acts as a dominant-negative form of Ap in the wing (O'Keefe et al., 1998), did not cause any phenotype in the legs (not shown). Only stronger and more sustained overexpression of *UAS-Chip* under the control of the *DllGal4* driver compromises the development of tarsus four, without affecting *ap* expression (Fig. 1G,H). However, Ap and Chip proteins seem to be associated in tarsus four, as a chimaeric Ap-Chip protein that acts as a functional Ap protein in the wing (van Meyel et al., 1999) also rescues the *ap* leg mutant phenotype (Fig. 2C), whereas an Ap fragment without the LIM domains does not (data not shown). Altogether these results indicate that although interaction between Ap and Chip is required for the development of the leg, their relative stoichiometry is not as crucial as it is in the wings, but rather is more similar to the situation in the CNS.

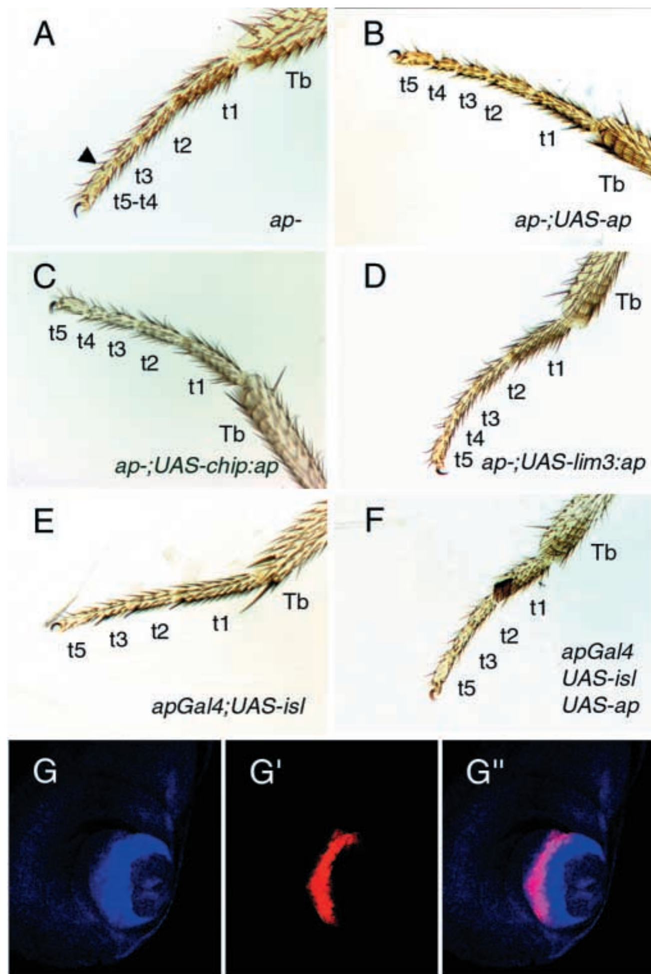

Fig. 2. Genetic interactions between LIM-HOM proteins and Ap function. (A) Leg of an *apGal4/ap*^{UGO35} fly. The fourth tarsal segment is almost completely lost and is fused to the fifth (arrowhead), whereas the other leg segments are normal. (B) Rescue of the *apGal4/Df(2)nap1* leg mutant phenotype by overexpression of an *ap* transgene. The *ap* mutant phenotype is completely rescued by one copy of the *UAS-ap* construct (compare with A and Fig. 1A). (C) Rescue of an *apGal4/Df(2)nap1* leg mutant phenotype by overexpression of the chimaera protein Chip-Ap, consisting of Chip lacking the LIM interaction domain (LID) linked to Ap lacking the LIM domains. (D) Leg of an *apGal4/Df(2)nap1;UAS-Lim3:ap-HD* fly. Ectopic expression of a chimaera protein, which consists of the LIM domains of Lim3 and the Ap homeodomain, rescues the *ap* mutant phenotype. (E) *apGal4;UAS-isl* leg lacking the fourth tarsal segment. (F) Leg of an *apGal4/UAS-ap;UAS-isl* fly. Overexpression of Ap does not overcome the dominant-negative effect produced by ectopic Islet expression (compare with E). (G-G″) Leg imaginal disc of an *apGal/UASGFP;UAS-islet* larva. Ectopic expression of Islet does not affect either Ap or Bar expression. (G) Bar protein distribution (blue). (G′) *apGal4* expression (red). (G″) Merged image.

It has been previously reported that *dlmo* is expressed in the legs, but its pattern of expression has not been fully characterised (Zeng et al., 1998). Using anti-Dlmo antibody and a *dlmoGal4* reporter line (Milan et al., 1998), we did not detect Dlmo expression in the leg tissue but in a few cells of the peripodial disc membrane (Fig. 1D). In addition, loss-of-function *dlmo* alleles did not produce a mutant leg phenotype (not shown). Another *dlmo*-like gene annotated as CG5708 has been found in the fly genome (Adams et al., 2000). In situ hybridisation was performed using a specific cDNA for this gene as a probe and expression was observed in the CNS, but not in the leg imaginal discs (not shown). Thus it appears that *dlmo* genes do not regulate LIM-HOM function in the legs. Nevertheless, ectopic expression of *UAS-dlmo* in the *ap* domain causes the loss of the fourth tarsal segment (Fig. 1I) without removing Ap protein expression (data not shown). As the Dlmo protein cannot bind the LIM domains of Ap but does bind the Chip LIM-interaction domain with higher efficiency than Ap (Milan et al., 1998; Weihe et al., 2001), it is possible that ectopic expression of Dlmo in the leg sequesters Chip, thereby disrupting the formation of Chip-Ap complexes. In agreement with this interpretation, partial rescue of the *UAS-dlmo* dominant-negative effect was achieved by co-expression of *UAS-Chip* (Fig. 1J). Therefore, although the *dlmo* gene is not expressed during the development of the wild-type leg, its ectopic expression interferes with the posttranslational interaction of Ap and Chip. We used a similar rationale to identify further interacting partners of Ap and Chip in the legs.

Ectopic expression of LIM-HOM genes interferes with Ap function posttranslationally

Sequence comparisons have shown that LIM-HOM proteins have been conserved throughout evolution (Dawid et al., 1998; Hobert and Westphal, 2000). Their developmental role also seems to be conserved, because distinct neural fates are specified by identical combinations of LIM-HOM genes in *Drosophila* and in vertebrates (Thor et al., 1999). Furthermore, ectopic expression of vertebrate LIM-HOM orthologues induce the same developmental effects in flies as the endogenous *Drosophila* genes do, indicating that the mechanisms of action of LIM-HOM proteins are conserved (Rincón-Limas et al., 1999; Tsuji et al., 2000). In view of these LIM-HOM functional relationships, we tested whether other LIM-HOM proteins could rescue Ap function in legs. Expression of *UAS-Lim1* in *ap* mutant legs does not produce

any rescue of the *ap* mutant phenotype (Pueyo et al., 2000), and no rescue was obtained by expressing the LIM-HOM gene *islet* either (not shown). However, O'Keefe et al. (O'Keefe et al., 1998) showed that expression of a hybrid protein containing the LIM domains of Lim3 and the homeodomain of Ap (Lim3-Ap) was able to partially rescue the *ap* mutant phenotype in the wing. This functional overlap extends to the leg as the hybrid Lim3-Ap molecule also rescues the *ap* mutant leg phenotype (Fig. 2D); this shows that the primary function of the LIM domains of Ap must be common to those of Lim3, most likely binding of Chip as shown in other systems (Milan et al., 1998; Thor and Thomas, 1997; van Meyel et al., 2000).

When LIM-HOM genes (*Lim3, islet*) were expressed ectopically in the *ap* domain of otherwise wild-type flies, the existence of an unknown cofactor of Ap was revealed. *apGal4;UAS-Lim3* and *apGal4;UAS-isl* flies lack the fourth tarsal segment (Fig. 2E). These flies still expressed *ap* in the legs (Fig. 2G-G″) and their phenotype was not rescued by simultaneous co-expression of *UAS-ap* (Fig. 2F). As these LIM-HOM proteins can interact with Chip, quenching of Chip

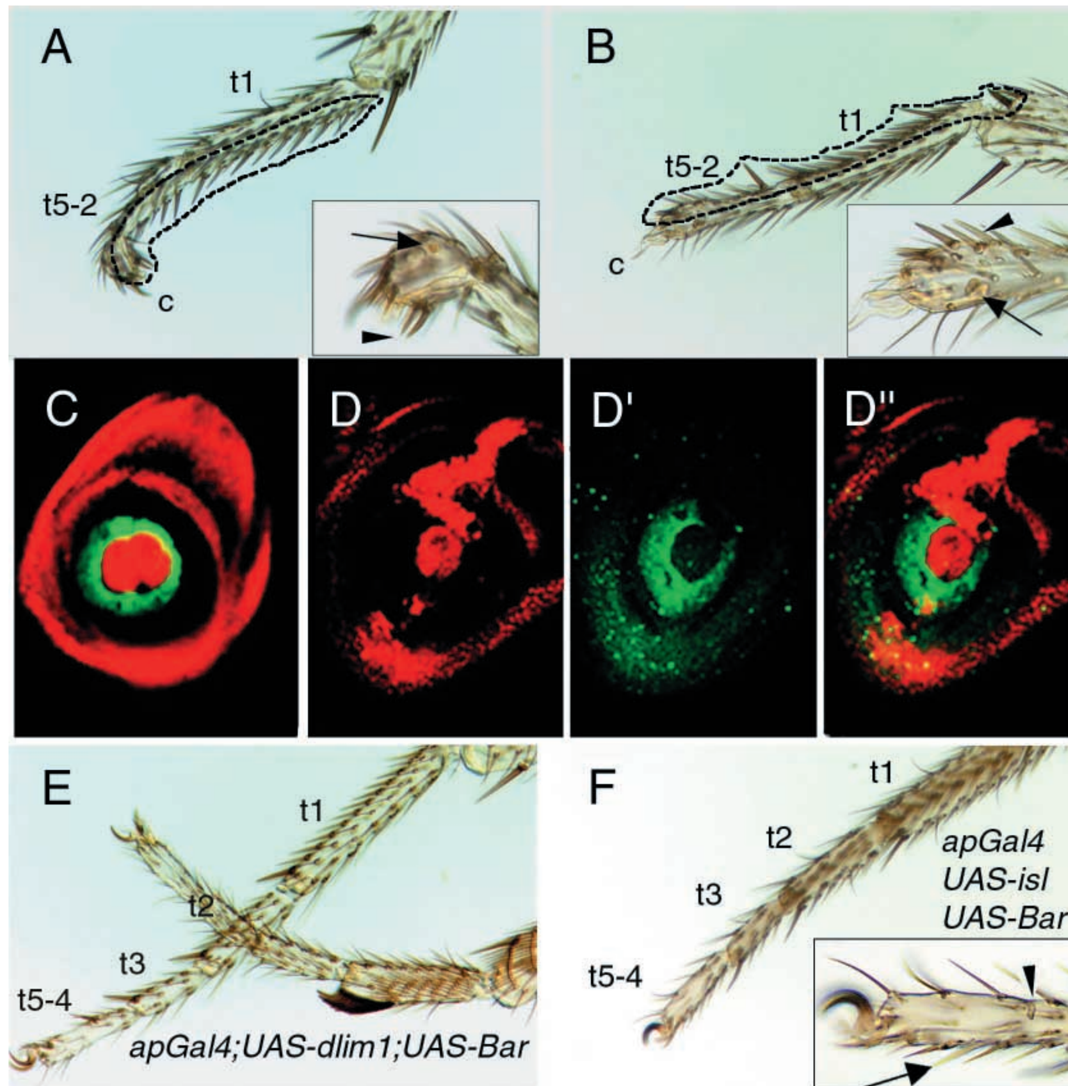

Fig. 3. Bar is the factor affected by ectopic LIM-HOM protein expression in the fourth tarsal segment. (A) Leg with *Bar* mutant clones marked by *forked* phenotype in the tarsal region. A large clone along the ventral (lower) side of the leg is outlined in black. Cells lacking *Bar* do not grow properly and the tarsal segments t2-t5 appear fused. Magnification of the distal part of the clone is shown in the inset, showing a remnant joint (arrow) and a *forked* bristle (arrowhead). (B) Leg with *Bar* mutant clones similar to those shown in A, except that here they also express Ap using *DllGal4* (see Materials and methods). The distal tarsal segments are fused, similar to those observed in *Bar* clones. Inset shows a magnification of the distal part of the clone, showing a *forked* bristle (arrowhead) and a remnant joint (arrow). (C) Leg imaginal disc showing *Bar-lacZ* reporter expression in a ring of cells in the presumptive fourth and fifth tarsal region (green), and Lim1 distribution (red). (D-D″) Ectopic expression of the *Lim1* gene represses Bar in a *dppGal;UAS-Lim1* leg imaginal disc. (D) Lim1 protein distribution. (D′) Bar protein distribution, showing an absence of Bar in the area where Lim1 is present. (D″) Merged image. (E) Leg of an *apGal4/UAS-Lim1;UAS-Bar* fly. Overexpression of Bar in an *apGal4/UAS-Lim1* genetic background partially rescues the *ap*-like dominant-negative effect of ectopic Lim1 (Pueyo et al., 2000). (F) Overexpression of Bar in an *apGal4;UAS-islet* genetic background also partially rescues the loss of the fourth tarsal segment (compare with Fig. 2E). Magnification of the distal part is shown in the inset. A remnant joint in the dorsal part of the fused t4-t5 segment is seen (arrowhead). An apical bristle can also be distinguished (out of focus; arrowhead).

could explain their *ap*-like dominant-negative phenotypes. However, these dominant-negative effects were also not rescued by co-expression of *UAS-Chip* (data not shown). The lack of phenotypic rescue by co-expression of either Chip or Ap could be due either to higher binding affinities of the Islet and Lim3 proteins, or to different levels of expression of the UAS transgenes employed. However, several *UAS-ap* and *UAS-Chip* transgenes with different expression levels were used in these experiments, with no rescue of the dominant-negative mutant phenotype. An alternative explanation is that Lim3 and Islet proteins interfere with another cofactor required for Ap function in the legs.

Overexpression of *Bar* suppresses the dominant-negative effect caused by ectopic LIM-HOM proteins in the *ap* domain

An element related to Ap function is the HOM gene *Bar*. *Bar* is required for the development of tarsal segments four and five (Fig. 1B,B′ and Fig. 3A) (Kojima et al., 2000). The main functional role of Bar in tarsus four had been attributed to the transcriptional activation of *ap*, as part of the regulatory network patterning the distal leg (Kojima et al., 2000; Pueyo et al., 2000). Ectopic Lim1 driven by *apGal4* eliminates tarsal structures and represses Bar expression (Fig. 3C,D-D″), leading to the loss of Ap expression (Pueyo et al., 2000). As it might be expected, co-expression of Bar in *apGal4/UAS-Lim1;UAS-Bar* flies produces a partial rescue of the fourth tarsal segment (Fig. 3E).

However, the dominant-negative effect on tarsus four produced by ectopic expression of Islet is not mediated by repression of either Bar or Ap expression (Fig. 2E,G-G″). Surprisingly, this Islet effect was also partially suppressed by co-expressing Bar (Fig. 3F). Therefore, Bar may be the Ap cofactor in tarsus four that is interfered with by ectopic Islet and Lim3 proteins, and whose existence we inferred in the previous section. To confirm whether there is a requirement for Bar in fourth tarsal development apart from *ap* transcriptional activation, we generated *Bar* mutant clones that still express *ap* in the distal part of the leg (see Materials and methods). *Bar* mutant clones show, as described before, a fusion of T3 to T5 tarsal segments (Fig. 3A) (Kojima et al., 2000). When the *UAS-ap* transgene was expressed in these *Bar* mutant clones, no phenotypic rescue was observed (Fig. 3B), indicating that the functional requirement for Bar in tarsus four goes beyond the activation of *ap*, and favouring its role as an Ap cofactor.

A proper balance of Bar and Ap proteins is required during tarsal development

LIM-HOM proteins can form multi-protein complexes with other HOM proteins, either by direct interactions or through interaction with Ldb proteins (Hobert and Westphal, 2000). As Bar behaves as a cofactor of Ap, Ap and Bar proteins could interact and form a transcriptional complex to regulate target genes. In this case, changes of dosage of either *Bar* or *ap* might disrupt the formation of Ap-Bar complexes. To test this hypothesis, we performed gene dosage experiments. First, the phenotypes of both *Bar* (InB^{M2}) and *ap* ($apGal4/ap^{UGO}$ at 25°C) mutants were enhanced by removing a copy of *ap* and *Bar*, respectively (Fig. 4A-C; compare with Fig. 2A). Second, overexpression of Bar causes loss of the fourth tarsal segment (Fig. 4E), although Ap was still expressed in these flies (Fig. 4D) (Kojima et al., 2000). As Bar is expressed in a graded manner in the wild type, at a higher level in the fifth tarsal segment and at a lower level in the fourth (Fig. 1B,B′), these observations suggest that the correct amount of Bar is necessary for the development of the fourth tarsal segment. If Bar overexpression alters the stoichiometry of Ap and Bar, and thus prevents the formation of functional Ap-Bar complexes, then this dominant-negative effect should be rescued by restoring the appropriate balance with co-expression of Ap. As predicted, *apGal4/UAS-ap;UAS-Bar* flies show a completely rescued phenotype with five tarsal segments (Fig. 4F).

No rescue of the dominant-negative effect of *UAS-Bar* on tarsus four was obtained by co-expression of Ap protein fragments lacking the LIM domains or the homeodomain (data not shown), which suggests that an Ap protein with functional LIM and HOM domains is required for the rescue. Interestingly, full rescue of the Bar overexpression phenotype by co-expression of the chimeric Lim3-Ap protein was observed (Fig. 4G), indicating again a non-specific requirement for the LIM domains. The LIM domains could be interacting with a common cofactor, such as Chip, in the formation of Ap-Bar functional complexes. In agreement with this interpretation, the dominant-negative effect caused by *UAS-Bar* overexpression was also completely rescued by simultaneous overexpression of *UAS-Chip*, revealing that Chip is involved in the interaction between Ap and Bar (Fig. 4H). This hypothesis is also supported by the requirement for Chip in tarsus four development (Fig. 1E,F), and by the dominant-negative effect of *UAS-Chip* overexpression with strong *Gal4* drivers (Fig. 1G). In addition, no rescue of the *UAS-Bar* dominant-negative phenotype was obtained by co-expression of Chip protein fragments lacking the dimerisation domain or the LIM-interacting domain (Fig. 4I), suggesting that both domains of Chip are required for the rescue of this *UAS-Bar* dominant-negative effect. Finally, to determine whether the interaction of Ap with Bar is carried out through an Ap-Chip dimer, the chimaeric Chip-Ap fusion protein was co-expressed with *UAS-Bar*. Partial rescue of the dominant-negative phenotype was observed (Fig. 4J), suggesting that Ap and Chip bind to each other to form functional protein complexes and interact with Bar in the fourth tarsal segment.

Bar interacts with Chip and Ap

To test the possibility of Chip-mediated complexes involving Bar, a direct interaction between Bar and Chip was tested in a Gst pull-down assay. Bar protein, both expressed in vitro or present in leg disc extracts, is retained by Chip-Gst fusions (Fig. 5A,B), as is Lim1 (Fig. 5A,B) (Lilly et al., 1999) and Ap (Torigoi et al., 2000). This protein interaction explains the requirement for Chip in tarsus five, and suggests that a complex of Bar-Chip is the functional element in this segment. In tarsus four, Chip seems to also bind Ap, as shown by the results involving the Ap-Chip chimaera in the legs (see above). Therefore, the dominant-negative interactions between Bar and Ap (and other LIM-HOM) proteins in tarsus four could be based on competition for Chip.

However, whereas the interaction between Chip and Ap or Lim1 depends on the LIM-interaction domain (LID) of Chip (Fig. 5A,B), and the interaction of Chip with other HOM proteins, such as Bicoid, depends on the Other Interaction Domain (OID) (Fig. 5A) (Torigoi et al., 2000), the interaction

between Chip and Bar does not rely on these domains (Fig. 5A,B). This suggests that, in principle, binding of Bar and Ap to Chip does not need to be mutually exclusive. These results are compatible with the possibility of Bar and Ap being simultaneously bound to Chip in a complex. If this were the case, an association between Ap and Bar proteins could be found. As expected, Bar protein present in protein extracts from leg discs is retained by Gst-Ap in a pull-down experiment (Fig. 5C,D). A fragment of Ap consisting of the LIM domains is also able to pull down Bar in a similar experiment (Fig. 5C,D). Altogether these results suggest that the interaction between Ap and Bar takes place in a protein complex that involves Chip (see Discussion).

In summary, our results show that the development of the tarsus requires stoichiometric interactions between Bar, Ap and Chip proteins, with Bar being the limiting factor in this process. These interactions seem to rely on: (1) the binding of Ap and Chip through their LIM and LID domains, respectively; (2) an interaction between Bar and Chip through a different domain; and (3) further complexing mediated by the Chip dimerisation domain. These interactions lead to the formation of Ap-Chip-Bar and Bar-Chip, functional units in tarsus four and five, respectively. Direct interactions between LIM-HOM and HOM transcription factors leading to the transcriptional regulation of target genes have already been described (Bach et al., 1997).

Interactions between LIM-HOM, Chip and HOM proteins in the pretarsus

The pretarsus at the tip of the leg is composed of the claw organ (a multicellular organ providing sensory information and grip to the substrate), plus a muscle attachment site and its associated tendon. *Lim1* and the Prd-HOM gene *al* are required for pretarsus development, and display synergistic functional interactions (Pueyo et al., 2000). One of the outcomes of their co-operation is the repression of Bar expression. Thus, weak alleles of *al* or strong alleles of *Lim1* lead to mild ectopic Bar expression in the pretarsus (Fig. 6A,B) (Kojima et al., 2000), whereas complete loss of both *al* and *Lim1* allows Bar to completely invade the presumptive leg tip (Fig. 6C) (Tsuji et al., 2000). Reciprocally, ectopic expression of Al or Lim1 alone does not repress Bar (Fig. 6D) (Kojima et al., 2000), but ectopic expression of Lim1 plus the ensuing ectopic expression of Al (Fig. 6F) (Tsuji et al., 2000) produce loss of Bar expression (Fig. 3D-D″) (Pueyo et al., 2000). The repression of Lim1 plus Al on Bar expression is reciprocal, as ectopic

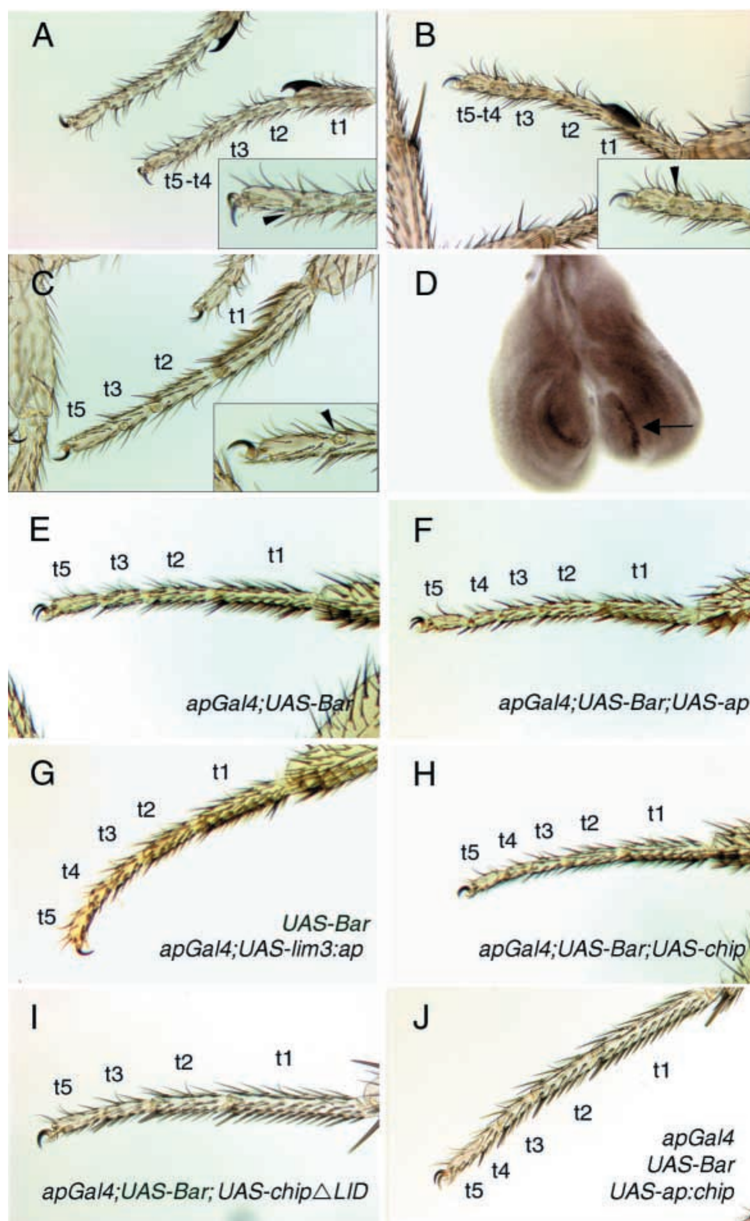

Fig. 4. Bar is the limiting factor for the development of the fourth tarsal segment. (A) Leg of an *InB*^{M2} mutant. This mutation produces partial loss of Bar function (Kojima et al., 2000). In these mutants, 33% of the legs show a weak fusion between the fourth and fifth tarsal segment, with the joint not properly differentiated (inset, arrowhead). (B) Leg of a *InB*^{M2}; *ap*^{UGO} /+ mutant. Removal of a copy of *ap* enhances the mutant phenotype observed in *InB*^{M2} mutants. Tarsus four and five are shorter and are fused in 61% of the legs (inset, arrowhead; compare with A). (C) The *ap* mutant phenotype is also enhanced by reducing Bar function in *InB*^{M2}; *ap*^{UGO} /*apGal4* flies (compare with Fig. 2A). Tarsus four is completely absent (inset), and even the joint between tarsus five and three is affected (arrowhead). (D) Overexpression of Bar does not repress Ap expression in the fourth tarsal segment (arrow). (E) Leg of an *apGal4;UAS-Bar* fly. Overexpression of Bar prevents the development of the fourth tarsal segment. (F) Overexpression of Ap rescues completely the phenotype caused by overexpression of Bar in the *ap* domain (compare with E). (G) Ectopic expression of a hybrid molecule, consisting in the LIM domains of Lim3 and the Ap homeodomain, completely rescues the loss of tarsus four phenotype produced by overexpression of Bar (compare with E). (H) Rescue of the *apGal;UAS-Bar* phenotype is also achieved by overexpression of Chip. (I) Ectopic expression of Chip lacking the LIM interaction domain is not able to rescue the dominant-negative effect produced by Bar overexpression. (J) Ectopic expression of the Chip-Ap hybrid protein partially rescues the *apGal4;UAS-Bar* phenotype, suggesting that Ap interacts with Chip to form dimers in tarsus four. However, the hybrid protein does not rescue as efficiently as the Ap and Chip wild-type proteins (compare with F, G and H).

Bar represses Al and Lim1 expression (Fig. 6I-I‴; compare with H-H‴) (Tsuji et al., 2000), producing the loss of pretarsal structures (Fig. 6E), whereas loss of Bar leads to ectopic expression of Lim1 (Fig. 6G). Thus, mutual antagonism between Al plus Lim1 in the pretarsus and Bar in the tarsus leads to mutually exclusive patterns of expression, and establishes a sharp pretarsus-tarsus boundary that is crucial for both tarsus five and claw organ development (Kojima et al., 2000; Pueyo et al., 2000).

As Al, Lim1 and Bar are transcription factors, these regulatory interactions could be directly and solely mediated by binding to their respective regulatory regions. However, we have also uncovered functional interactions not based on transcriptional control. When Al is ectopically expressed in the *ap* domain in *apGal4/UAS-al* flies, neither the expression of Bar or Ap is affected (not shown), yet a loss of tarsus four is produced (Fig. 7A). This dominant-negative effect is partially rescued by co-expression of Bar (Fig. 7B), but not by expression of Ap or Chip, indicating that, similar to LIM-HOM proteins, Al can exert a posttranslational effect on Bar. Reciprocally, ectopic expression of the LIM-HOM proteins Ap, Islet or Lim3 in the pretarsus leads to loss of claw organ

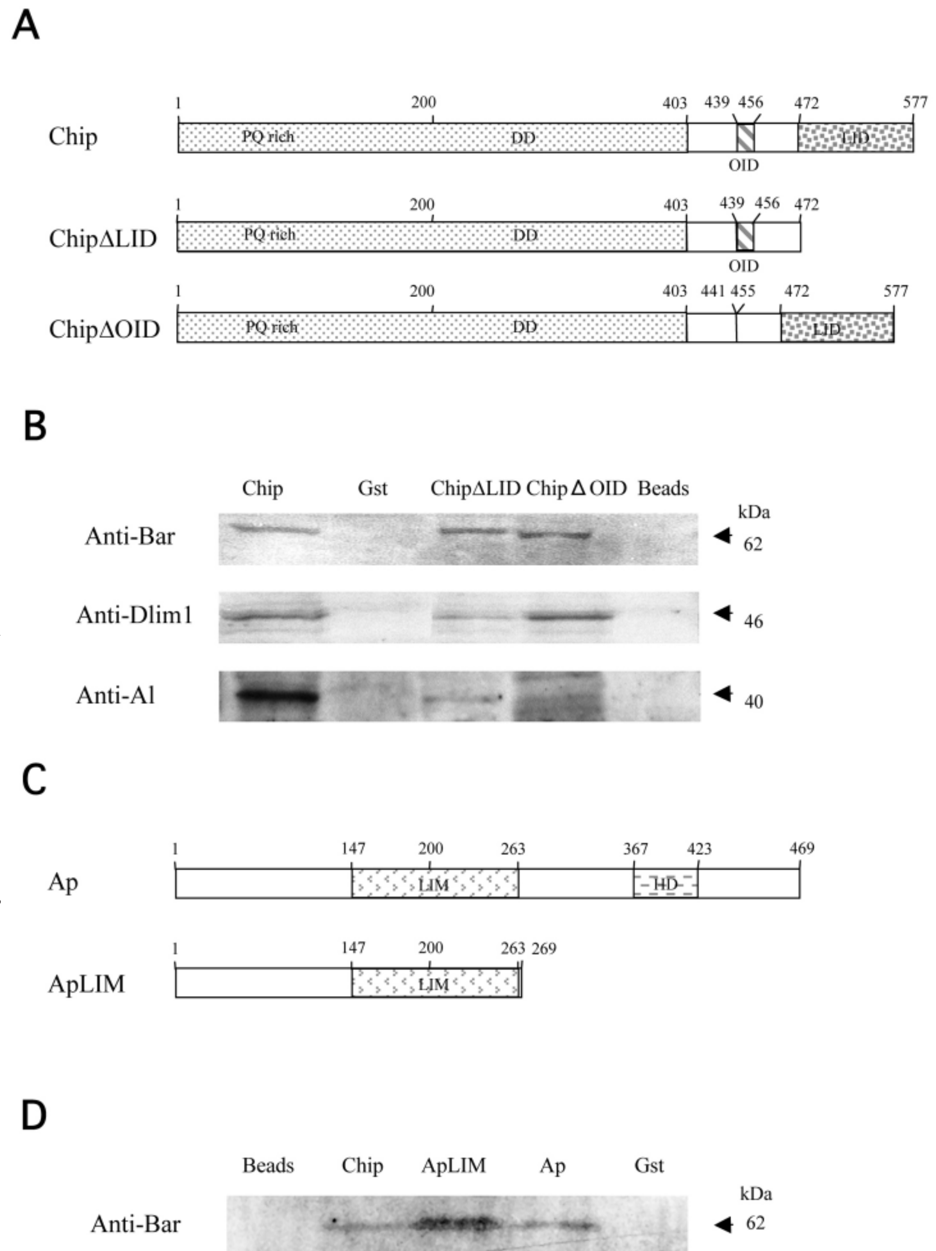

Fig. 5. Bar interacts with the Chip and Ap proteins. (A) Representation of different domains in Chip and deleted Chip proteins. Chip contains a proline and glutamine rich (PQ rich) region at the amino-terminal end, followed by a Dimerisation Domain (DD). The LIM interaction domain (LID) is located at the carboxyl-terminal end. The Other Interaction Domain (OID) appears between amino acids 439 and 456, and mediates the interaction with Bicoid. The ChipΔLID protein lacks the LIM interaction domain, and the ChipΔOID lacks the OID domain. (B) Sample western blots of the affinity chromatography experiments using leg disc extracts; Gst-Chip fusion proteins and beads used are indicated at the top of the lanes, and the different antibodies used for immunodetection are indicated on the left. The 'Gst' and 'Beads' lanes show the lack of protein retained by beads with the Gst protein alone, and by the Gluthathione-agarose beads alone, respectively. Other lanes on the top row show an ~62 kDa band in the anti-Bar western blot, corresponding with the predicted size of Bar. Bar is able to interact with Chip, and with the Chip LID- and Chip OID-deleted proteins, but it does not interact with Gst or with beads alone. Similarly, in the middle row a ~46 kDa band is detected in the anti-Lim1 western blot, showing that Chip interacts with Lim1. However, a decrease of signal of this band is detected in the ChipΔLID lane, as has also been found with Ap (Torigoi et al., 2000), corroborating that the LID is crucial for the interaction between Ldb and LIM-HOM proteins. The lack of the OID domain does not affect this interaction. Finally, in the bottom row the western blot shows that Al interacts with Chip. An ~40 kDa band corresponding with the predicted size of Al is detected. A decrease of the signal is observed in the ChipΔLID lane and the signal is almost undetectable in the ChipΔOID lane. Thus, both protein domains are necessary for the proper binding of Al. (C) Representation of the protein domains in Ap and Ap-LIM proteins. The Ap protein contains two LIM domains at the amino-terminal part of the protein followed by a homeodomain. The Ap-LIM protein consists of the amino-terminal end containing the LIM domains. (D) Western blot carried out similar to that shown in B, but with Gst-Ap constructs. The same 62 kDa band was detected using the anti-Bar antibody. Bar interacts with Ap and Ap-LIM, as well as with Chip, but it does not interact with Gst or with beads alone. The increase of signal in the Ap-LIM lane in comparison with in the Ap and Chip lanes is due to the higher molarity of Ap-LIM protein loaded in comparison with Ap and Chip proteins.

elements without affecting the expression of Lim1 (Fig. 7C-D′). We surmise that the functional relationship in the wild-type pretarsus between Al and Lim1 may be similar to that of Bar and Ap in tarsus four, i.e. putative protein interactions leading to the formation of multimeric transcriptional complexes. In agreement with this hypothesis, Chip binds to the LIM domains of Lim1 (Fig. 5A,B) (Lilly et al., 1999) and is required for pretarsus development (Fig. 1E,F). Strong overexpression of Chip in the pretarsus also leads to *Lim1*-like mutant phenotypes (Fig. 1G), mimicking the results obtained in tarsus four. Supporting this hypothesis, ectopic expression of Dlmo in the pretarsus also produces loss of claw organ without affecting Lim1 expression (Fig. 7F,H,I). Finally, overexpression of Lim1 in the pretarsus also has a dominant-negative phenotype, just as Bar does in the fourth tarsal segment (Fig. 7G). It follows that, as in the fourth tarsal segment, Chip might be participating in direct interactions between LIM-HOM and Prd-HOM proteins. This putative interaction with Chip seems to involve the LIM-interaction and the dimerisation domains, as ectopic expression of a Chip fragment lacking either of these domains produces loss of the claw organ (Fig. 7E), possibly by still being able to sequester Lim1 (Fig. 5A,B).

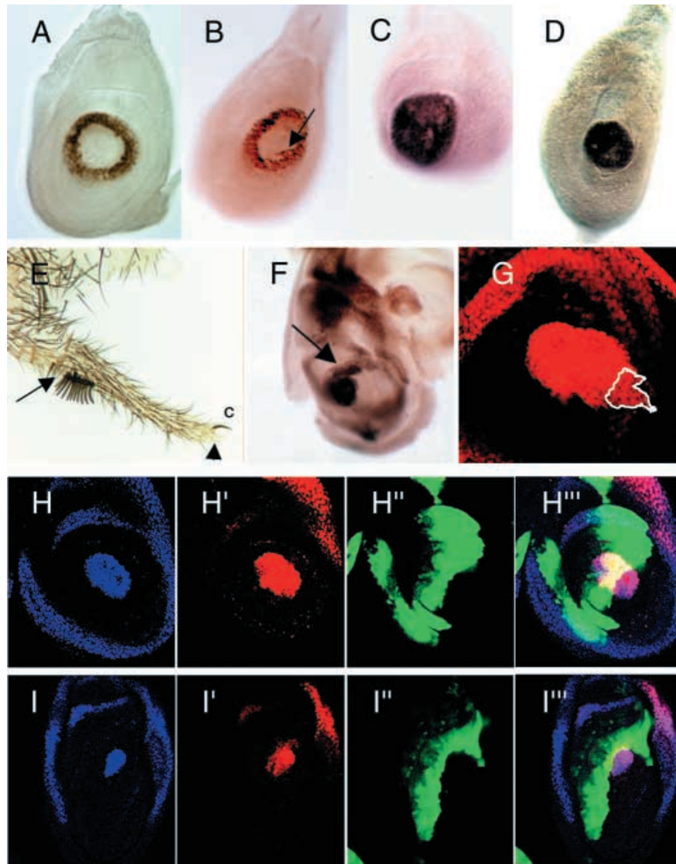

Fig. 6. Genetic relationships between the tarsal gene *Bar* and the pretarsal genes *al* and *Lim1*. (A) Pattern of expression of a reporter *Bar-lacZ* in a wild-type late third instar leg imaginal disc. (B) A $Lim1^{R12.4}$ mutant leg imaginal disc showing *Bar-lacZ* reporter expression invading the pretarsal region (arrow, compare with A). (C) *Bar-lacZ* expression in an *al* strong mutant (al^{ex}/al^{ice}), which lacks *al* and loses *Lim1* expression (Pueyo et al., 2000). *Bar* expression invades the whole pretarsal region at the centre of the disc. (D) *Bar-lacZ* expression in an al^{ice}/al^{ex} mutant background expressing Lim1 driven by the *dppGal4* driver. Expression of Lim1 is not able to repress *Bar* expression in the absence of Al function (compare with Fig. 3C,D-D″). (E) Leg from a *dppGal4/UAS-Bar* fly. Ectopic expression of Bar produces fusion of the proximal segments, such as femur, tibia and the first tarsal segment (arrow), and in the pretarsus one claw is missing (arrowhead). (F) Ectopic Al expression (arrow) produced by *dppGal;UAS-Lim1*. (G) High magnification of the pretarsal region from a leg imaginal disc with a clone of cells deficient for *Bar* (outlined in white). Lim1 expression (red) extends into the clone. (H-H‴) A *dppGal4/UAS-GFP* leg disc showing Lim1 protein in blue (H), Al protein in red (H′), and the pattern of *Gal4* expression in green (H″), in an otherwise wild-type leg. (H‴) Merged image. (I-I‴) A *dppGal4/UAS-GFP;UAS-Bar* leg disc stained as in H. Ectopic expression of Bar represses Lim1 and Al in the pretarsus (compare with H).

To test this hypothesis, a Gst pull down using different Gst-Chip constructs was performed. Al protein from leg disc extracts is retained by the full-length Chip construct, suggesting that a direct protein interaction exists (Fig. 5A,B). The Al interaction with the two other Chip deletion constructs is weaker or absent, suggesting that both domains are required for the proper binding between Chip and Al (Fig. 5A,B). Altogether our results support the hypothesis of a balanced functional relationship between Lim1, Al and Chip that can interfere with, or be interfered by, other LIM-HOM proteins, and that might be based on multimeric, specific protein complexes.

Discussion

Biochemical studies in vitro have shown that LIM-HOM transcription factors confer little transcriptional activation of target genes on their own (Bach et al., 1995; German et al., 1992). LIM-HOM proteins interact with a variety of proteins, including members of the bHLH family (Johnson et al., 1997), the POU family (Bach et al., 1995), the PAS family (Bach et al., 1997) and also other LIM family members (Jurata et al., 1998; Thaler et al., 2002; Thor et al., 1999), to control specific developmental processes (Hobert and Westphal, 2000). It has been suggested that these protein interactions confer specificity and modulate LIM-HOM activity (Bach, 2000). For example, Dlmo proteins reduce LIM-HOM activity, and Lbd proteins such as Chip modulate LIM-HOM activity by acting as a bridge between LIM-HOM proteins and Chip-binding cofactors, thus leading to the formation of heteromeric complexes. An example of regulation of LIM-HOM protein activity in different contexts is the development of *Drosophila*.

A regulatory network of transcription factors controls distal leg development

Bar and *ap* genes are expressed in the fourth tarsal segment and are required for its proper development, whereas the *al* and *Lim1* genes are expressed and required in the pretarsus (Kojima et al., 2000; Pueyo et al., 2000; Tsuji et al., 2000). All of these genes encode putative transcription factors and display canonical regulatory relationships. Thus, *al* activates *lim1* expression and then both genes cooperate to repress *Bar* expression in the pretarsus. Reciprocally, Bar represses *al* and *lim1* expression while activating the expression of *ap* in tarsus four. After the refinement of their gene expression domains by

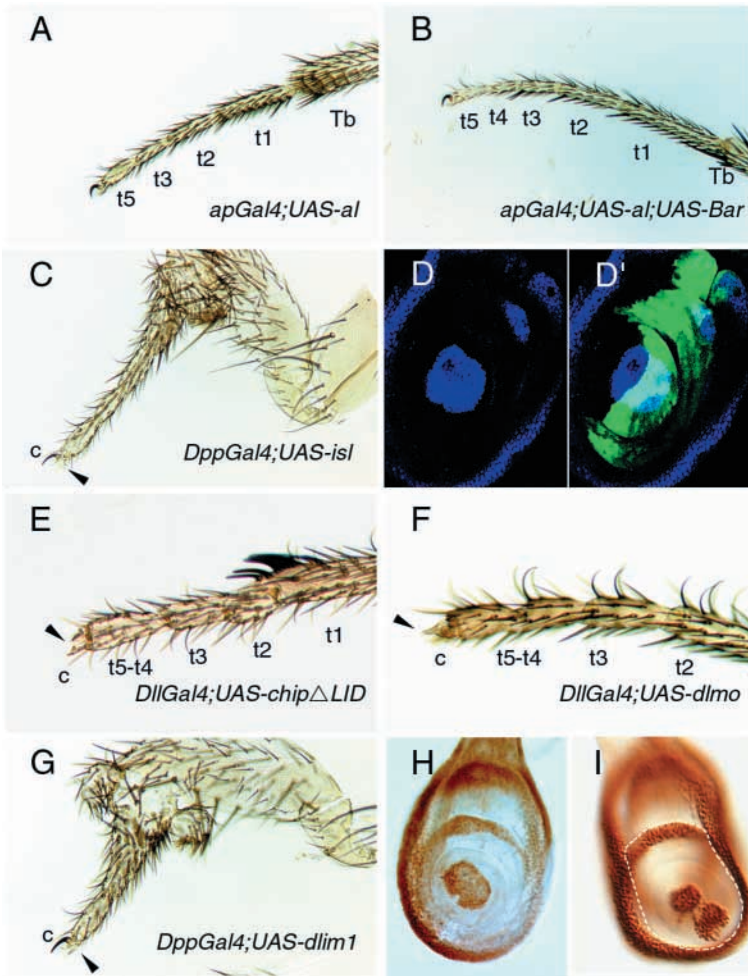

Fig. 7. Functional relationships between LIM-HOM, Prd-HOM, Chip and Dlmo proteins in the pretarsus. (A) Ectopic expression of Al using the *apGal4* driver produces a loss of the fourth tarsal segment. (B) Co-expression of Bar in an *apGal4/UAS-al* background partially rescues the dominant-negative effect on the development of the fourth tarsal segment (compare with A). (C) Ectopic expression of Islet using the *dppGal4* driver causes fusion of the femur, tibia and first tarsal segment. In the pretarsus only one claw develops (arrowhead). (D,D′) A *dppGal4/UAS-GFP;UAS-islet* leg disc showing Lim1 protein distribution (D, blue) and Lim1 protein distribution plus the *Gal4* pattern of expression (D′, green). Ectopic expression of Islet does not repress Lim1 expression; therefore the dominant-negative effect on Lim1 function seems to be posttranslational. (E) Ectopic expression of a truncated Chip protein lacking the LIM interaction domain with the *DllGal4* driver produces a similar phenotype in the pretarsus to that seen in *DllGal4;UAS-Chip* flies: lack of the claws (arrowhead), and fusion of the fifth and fourth tarsal segments (compare with Fig. 1G). (F) A *DllGal4;UAS-dlmo* leg. Ectopic expression of the *dlmo* gene mimics the *Lim1* lack-of-function phenotype. Arrowhead denotes the pretarsus lacking the claws. (G) Ectopic expression of Lim1 driven by the *dppGal4* driver disrupts leg development causing the fusion of femur, tibia, and tarsus one to three. In the pretarsus it produces a similar phenotype to that seen in *Lim1* mutants, or after ectopic expression of Lim1 antagonists (arrowhead; compare with C). (H) Pattern of Lim1 expression in a wild-type leg imaginal disc. (I) Lim1 protein distribution in a *DllGal4;UAS-dlmo* leg imaginal disc. The Lim1 protein is detected in a normal number of pretarsal cells, suggesting that the ectopic Dlmo effect on Lim1 function is not transcriptional, although part of the *Lim1* domain is disorganised (compare with H). The white dotted line denotes the proximal limit of the *DllGal4* pattern of expression.

these regulatory interactions, *Bar* directs tarsus five development, whereas cooperation between *al* and *lim1* directs pretarsus development (Pueyo et al., 2000), and cooperation between *Bar* and *ap* directs tarsus four (this study). Our results offer more evidence for the existence of this regulatory network, but also suggest an interesting role for direct protein interactions in its mechanism.

The cooperation between Bar and Ap on the one hand, and Al and Lim1 on the other, is likely to be carried out by transcriptional complexes involving Chip (Fig. 8). The Chip protein is required for development of the tarsus four, five and pretarsus, and Gst experiments reveal its ability to bind Ap, Bar, Lim1 and Al (Lilly et al., 1999; Milan et al., 1998) (this work). However, our results also show that modulation of LIM-HOM protein activity by Chip alone does not explain distal leg development. For example, Ap function is not modulated primarily by Chip and Dlmo. The relative amount of Chip and Ap has to be grossly unbalanced before a phenotype is obtained in the leg (Pueyo et al., 2000) (this work), and *dlmo* is not expressed or required in leg development. Furthermore, the interaction between Ap and Chip does not confer the developmental specificity that allows LIM-HOM proteins to produce different outcomes in different parts of the leg. First, Ap and Chip also interact in the wing and the CNS. Second, a chimaeric Lim3-Ap protein containing the LIM domains of Lim3 and the HOM domain of Ap does not behave as a dominant negative when expressed in tarsus four, and is even able to fulfil Ap function and rescue *ap* mutants. In the distal leg, developmental specificity seems to be achieved at the level of DNA binding and the transcriptional control of targets genes, mediated by partnerships between LIM-HOM and HOM proteins.

The evidence for this is presented first by dosage interactions between LIM-HOM and HOM proteins. Whereas there seems to be a relative abundance of endogenous Ap in tarsus four, an excess of Bar or Chip leads to a mutant phenotype, which is rescued by restoring the normal balance between Ap, Bar and Chip proteins in co-expression experiments. The effects observed could be explained simply by independent competition and the binding of Bar and Ap to Chip, leading, for example, to an excess of Bar-Chip complexes and a reduction of the pool of Chip available for Ap-Chip ones. However, this hypothesis alone does not explain the additional dominant-negative effects of ectopic LIM-HOM and HOM proteins in tarsus four (Lim3, Islet and Al), which are also not mediated by transcriptional regulation but are nonetheless rescued by co-expression of appropriate endogenous proteins. For example, ectopic expression of *UAS-islet* or *UAS-Lim3* in the *ap* domain produces loss of tarsus four without affecting Ap or Bar expression, and simultaneous co-expression of *UAS-Bar* partially suppresses this phenotype. If the sole effect of both *UAS-Bar* and *UAS-Lim3* or *UAS-islet* were to quench Chip away from Ap, then simultaneous co-expression of Bar and Lim3 or Islet should worsen the phenotype,

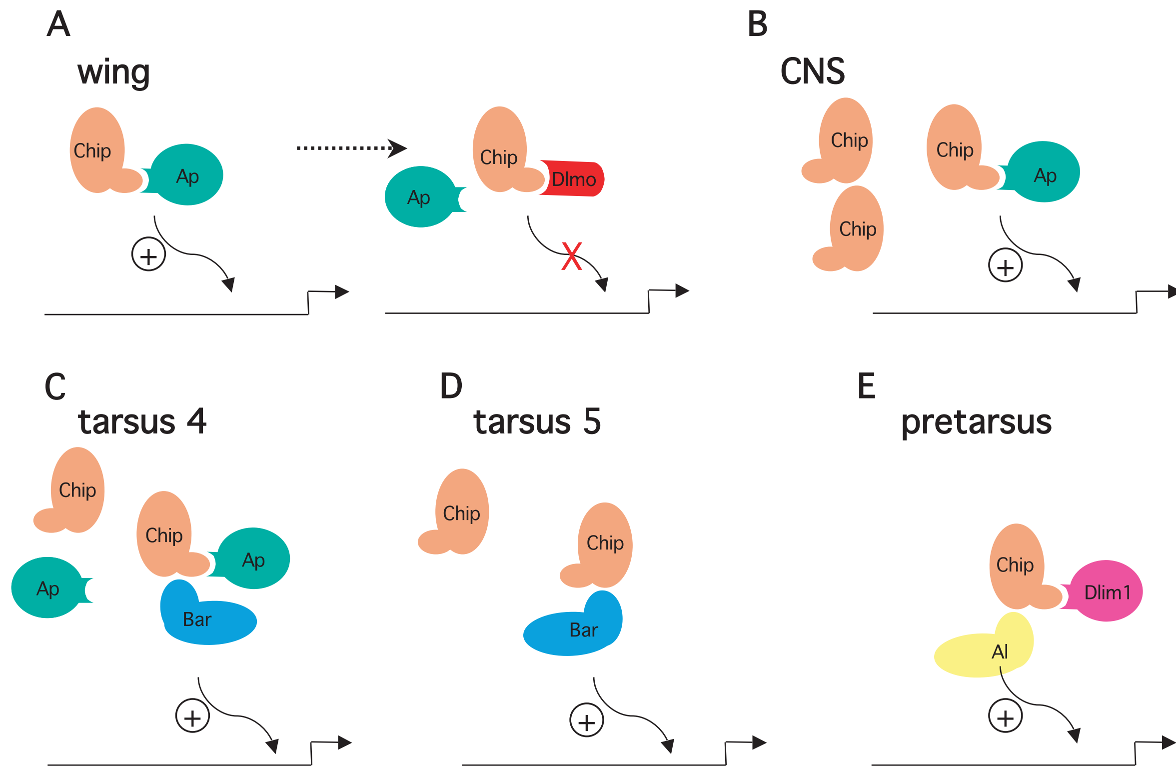

Fig. 8. Specific developmental functions are carried out by different partnerships between interacting LIM-HOM and HOM proteins. (A) Ap function in the wing is carried out by a complex of Ap and Chip. This unit dimerises to form a tetrameric complex comprising two molecules of Ap bridged by a Chip dimer. The relative stoichiometry of the two proteins is important for the formation of these complexes. Dlmo regulates Ap function by sequestering Chip into non-functional complexes. (B) Ap-Chip complexes are also necessary for the proper development of Ap motoneurones. However, balanced amounts of Chip and Ap are not required for tetrameric complex formation indicating that the limiting factor is Ap. In addition, there is no regulation by Dlmo. (C) In the fourth tarsal segment, Ap function might be achieved by a multiprotein complex, comprising Ap, Bar and Chip proteins. Our experiments indicate that the limiting factor in the formation of functional complexes is Bar, whereas Ap and Chip are more abundant. Bar interacts with Chip but not through the OID domain. This Ap-Chip-Bar functional unit could dimerise to produce a hexamer, or could consist of a molecule of each Ap and Bar bridged by a dimer of Chip. (D) High levels of Bar expression are required for the development of the fifth tarsal segment. As loss of Chip also affects the fifth tarsal segment, it is possible that a heterodimer of Bar and Chip is the functional unit in tarsus five. This unit could dimerise to produce a tetramer. (E) Synergism between Al and Lim1 is required for pretarsal development. Lim1 and Chip interact through their LIM and LID domains, respectively, and Al is also able to interact with Chip. In addition, genetic experiments show that Chip, Al and Lim1 are required in balanced amounts, suggesting that the functional unit in the pretarsus involves these three proteins simultaneously.

not correct it as observed. Moreover, ectopic expression of Islet or Lim3 proteins is not corrected by simultaneous co-expression of either *UAS-Chip* or *UAS-ap*. Altogether these results show instead that *UAS-islet* and *UAS-Lim3* must interfere posttranslationally with Bar. The most direct explanation is that Islet and Lim3 have the ability to quench Bar protein into a non-functional state. Interestingly, the hybrid *UAS-Lim3:ap* does not behave as dominant negative but as an endogenous Ap protein in these experiments, as it does not produce a mutant phenotype on its own and it rescues *UAS-Bar* overexpression. This suggests that the LIM domains are not very specific when it comes to interaction with Bar, and points to the involvement of a common LIM-binding intermediary such as Chip. These results suggest that a protein complex involving Ap, Chip and Bar is the correct functional state of these proteins in tarsus four, and deviations from this situation into separate Bar-Chip, Ap-Chip, or Bar-Chip-Lim3 or Bar-Chip-Islet complexes leads to a mutant phenotype.

The notion of a protein complex involving Ap, Chip and Bar together is also supported by the Gst pull-down assays. The domain of Chip involved in Ap binding, the LID, is not involved in Bar binding. However, the LID and the dimerisation domains of Chip are necessary to rescue the dominant-negative effect of *UAS-Bar* on tarsus four, suggesting a requirement for the formation of a complex with a LIM-HOM protein such as Ap. In agreement with this view, the Ap protein, and the LIM domains of Ap alone, are able to retain Bar protein in a Gst assay.

In the pretarsus, Al and Lim1 are possibly engaged in a partnership with Chip similar to that suggested for Ap, Chip and Bar. Synergistic cooperation between Al and Lim1 is required to direct pretarsus development and to repress Bar expression and function. Their cooperation entails a close functional relationship because a proper balance of Al, Lim1 and Chip is required, as is shown by the loss of pretarsal structures in *UAS-Chip* or *UAS-Lim1* flies. Ectopic expression of LIM-HOM proteins in the pretarsus also disrupts pretarsal development without affecting Lim1 and Al expression. The

possibility of direct protein interactions between Al, Lim1 and Chip is also suggested by the reciprocal ability of Al to interfere posttranscriptionally with Bar and Ap in tarsus four, and by the binding of Chip to Lim1 and to Al in in vitro experiments (Fig. 5) (Lilly et al., 1999).

Different developmental outcomes correlate with different sets of interacting proteins

Comparison of tarsal development with other developmental processes illustrates how LIM-HOM proteins are versatile factors to regulate developmental processes. It had been observed that the outcome of LIM-HOM activity depends on their developmental context. This context we can now analyse as being composed of the presence, concentration and relative affinities of other LIM-HOM proteins, Ldb adaptors, and other cofactors such as LMO proteins and HOM proteins (Fig. 8). We propose that the different developmental outcomes of LIM-HOM protein function could be due to the precise identity and dosage of cofactors available locally.

Ectopic expression experiments distort these contexts and lead to non-functional or misplaced LIM-HOM activities. In the wing, a finely balanced amount of functional Ap protein is modulated by Dlmo and Chip (Fig. 8A). Over-abundance of Chip stops the formation of functional tetramers in the wing but not in the CNS, where the relative amount of Ap, which is not modulated by Dlmo, is limiting for the formation of Ap-Chip functional complexes (Fig. 8B) (Fernández-Fúnez et al., 1998; Milan and Cohen, 1999; Milan et al., 1998; O'Keefe et al., 1998; van Meyel et al., 1999; van Meyel et al., 2000). In tarsus four (Fig. 8C), the Ap-Chip-Bar partnership is affected by experimentally induced over-abundance of Chip, presumably also because ectopic Ap-Chip tetramers typical of the CNS and the wing, and Bar-Chip complexes typical of tarsus five, are produced. Similarly, an excess of Bar might be interpreted by the cells as being a wrong developmental outcome, as high levels of Bar in the absence of Ap direct tarsus five development (Fig. 8D) (Kojima et al., 2000). Overexpression of Ap rescues this Bar dominant-negative effect, by restoring the relative amounts of Bar and Ap, which are determinant and limiting for tarsus four development. Finally, the dominant-negative effects produced by overexpression of either Chip or Lim1 in the pretarsus could either prevent the formation of Al-Chip-Lim1 complexes (Fig. 8E), or could favour the existence of Lim1-Chip complexes typical of the CNS (Lilly et al., 1999).

The wing and the CNS models have postulated that Ap function is carried out by an Ap-Chip tetramer; however, the molecular scenario might be more complex. A new component of Ap-Chip complexes, named Ssdp, has been identified and is required for the nuclear localisation of the complex (Chen et al., 2002; van Meyel et al., 2003). Thus it is possible that an Ap-Chip tetramer also contains two molecules of Ssdp. In addition, different types of Chip-mediated transcriptional complexes and different regulators have been identified in other developmental contexts, such as in sensory organ development and thorax closure, in which the GATA factor Pannier forms a complex with Chip and with the bHLH protein Daughterless. Heterodimers of this complex are negatively regulated by a protein interaction with Osa (Heitzler et al., 2003; Ramain et al., 2000). Thus, although our results indicate that in different segments of the leg there exist specific interactions between LIM-HOM, Chip and HOM proteins, the involvement of further elements in these multiprotein complexes is not excluded.

Partnership between Prd-HOM and LIM-HOM proteins in flies and vertebrates

Our results support a partnership between HOM and LIM-HOM proteins in the specification of distinct segments of the leg, and the results are compatible with Ap-Chip-Bar, Bar-Chip and Lim1-Chip-Al forming transcriptional complexes. Although the characterisation of the target sequences, followed by further biochemical and molecular assays, is necessary to study the transcriptional mechanism of these interactions, it has been shown that LIM-HOM proteins can interact specifically and directly with other transcription factors to regulate particular genes. For instance, mouse Lim1 (Lhx1) interacts directly with the HOM protein Otx2 (Nakano et al., 2000). In addition, the bHLH E47 transcription factor interacts with Lmx1, and both synergistically activate the insulin gene (Johnson et al., 1997). This interaction is specific to Lmx1, as E47 is unable to interact with other LIM-HOM proteins such as Islet (Johnson et al., 1997). Moreover, Chip is able to bind to other Prd-HOM proteins, such as Otd, Bcd and Fz, to activate downstream genes (Nakano et al., 2000; Perea-Gomez et al., 1999; Shawlot et al., 1999; Varela-Echavarría et al., 1996). Chip also complexes with Lhx3 and the HOM protein P-Otx, increasing their transcriptional activity (Bach et al., 1997). Our results reinforce the notion of Chip as a multifunctional transcriptional adaptor that has specific domains involved in each interaction.

Experiments in *Drosophila* have demonstrated a conservation of LIM-HOM activity at the functional and developmental level in the CNS between *Drosophila* and vertebrates (Thaler et al., 2002; Thor et al., 1999). In addition, xenorescue experiments have shown that the mechanism of action of Ap and its vertebrate homologue Lhx2 is very conserved in *Drosophila* wings (Rincón-Limas et al., 1999), whereas ectopic expression of dominant-negative forms of chick Lim1, Chip, Ap and Lhx2 mimic both Ap and Lhx2 loss-of-function phenotypes (Bach et al., 1999; Milan and Cohen, 1999; O'Keefe et al., 1998; Rodríguez-Esteban et al., 1998; van Meyel et al., 1999). The developmental role of Ap, Bar and Al in the fly leg, and their putative molecular interactions may also have been conserved because their vertebrate homologues Lhx2, Barx and Al4 are also co-expressed in the limb bud (Barlow et al., 1999; Qu et al., 1997; Rincón-Limas et al., 1999). We would expect that the interactions between the LIM-HOM and Prd-HOM proteins shown here represent a conserved mechanism to specify different cellular fates during animal development.

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