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Limb type-specific regulation of *bric a brac* contributes to morphological diversity

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SUMMARY

The insect antenna and leg are considered homologous structures, likely to have arisen via duplication and divergence from an ancestral limb. Consistent with this, the antenna and leg are derived from primordia with similar developmental potentials. Nonetheless, the adult structures differ in both form and function. In *Drosophila*, one conspicuous morphological difference is that the antenna has fewer distal segments than the leg. We propose that this is due in part to the variations in the regulation of *bric a brac*. *bric a brac* is required for joint formation, and loss of *bric a brac* function leads to fusion of distal antennal and leg segments, resulting in fewer total segments. Here, we address how *bric a brac* is regulated to generate the mature expression patterns of two concentric rings in the antenna versus four concentric rings in the leg. We find that *bric a brac* expression is activated early throughout most of the *Distal-less* domain in both antenna and leg and subsequently is restricted to the distal portion and into rings. Although *bric a brac* expression in the antenna and in all four tarsal rings of the leg requires Distal-less, only the proximal three tarsal rings are Spineless-dependent. Thus *bric a brac* is regulated differentially even within a single appendage type. The restriction of *bric a brac* expression to the distal portion of the Distal-less domain is a consequence of negative regulation by distinct sets of genes in different limb types. In the leg, the proximal boundary of *bric a brac* is established by the medial-patterning gene *dachshund*, but *dachshund* alone is insufficient to repress *bric a brac*, and the expression of the two genes overlaps. In the antenna, the proximal boundary of *bric a brac* is established by an antenna-specifying gene, *homothorax*, in conjunction with *dachshund* and *spalt*, and there is much less overlap between the *bric a brac* and the *dachshund* domains. Thus tissue-specific expression of other patterning genes that differentially repress *bric a brac* accounts for antenna-leg differences in *bric a brac* pattern. We propose that the limb type-specific variations in expression of *bric a brac* repressors contribute to morphological variations by controlling distal limb segment number.

Key words: *Distal-less*, *homothorax*, *dachshund*, *bric a brac*, *spalt*, *spineless*, Antenna, Leg, Wing, Appendage, Limb, Proximodistal

INTRODUCTION

Morphological diversity among animal appendages facilitates their many functions in moving, feeding and sensing the environment. In insects, the antenna is the primary olfactory and auditory organ while the thoracic legs serve mainly in terrestrial locomotion. Not only are different types of appendages unique in their forms, but there also exist many modified forms of the same appendage type. One feature of appendages that varies even among the insects is the number and morphology of their distal segments (Snodgrass, 1935). In the dipteran insect *Drosophila melanogaster*, the distal portion of the leg, the tarsus, is divided into five segments (t1-t5), while the homologous region of the antenna consists of only two segments plus part of a third (a4 and a5 plus distal a3) (Postlethwait and Schneiderman, 1971). By studying how limb segmentation is differently regulated in homologous limbs, we will gain insights into how morphological variations among appendages are achieved and into how they evolved.

Although several genes are known to be expressed in segmentally reiterated patterns in the antenna and leg, most of these are unlikely to be regulated differently between these appendage types. For instance, *Serrate*, *Delta* and *fringe*, which function in the Notch pathway, are expressed and required for the formation of all joints and are not being limited to homologous subdomains of antenna and leg (Bishop et al., 1999; de Celis et al., 1998; Mishra et al., 2001; Parody and Muskavitch, 1993; Rauskolb and Irvine, 1999). Other genes, such as *Bar* and *apterous* are similarly expressed in narrow proximodistal (PD) subdomains that correspond to homologous segments of the antenna and leg (Kojima et al., 2000; Larsen et al., 1996; Pueyo et al., 2000; Rincon-Limas et al., 1999; Tsuji et al., 2000). Thus although all of these genes contribute to limb segmentation, they probably do not contribute to the morphological differences between appendages.

In contrast, *bric a brac* (*bab*), which is required specifically for distal limb segmentation, is expressed in restricted PD subdomains and exhibits distinct expression patterns between antenna and leg. *bab* encodes a BTB/POZ domain protein necessary for normal development of most tarsal and distal antennal joints (Godt et al., 1993). *bab* mutations cause fusion of tarsal segments 2 through 5 (t2-t5) of the leg and of the fourth and fifth segments (a4, a5) and arista (ar) of the antenna (see Fig. 1A-D) (Godt et al., 1993). The mature *bab* pattern consists of two concentric rings in the distal antenna and in four concentric rings in the distal leg (see Fig. 2C′,F′). To understand how the antenna and leg develop different numbers of distal segments, we therefore have focussed on how *bab* becomes differentially expressed in the antenna and the leg.

bab expression in the leg is Distal-less (Dll)-dependent (Campbell and Tomlinson, 1998), and it has been proposed that the activation of *bab* by Dll in the leg is mediated by Spineless (Ss) (Duncan et al., 1998). *Dll* encodes a homeodomain transcription factor needed for the formation of the trochanter to the distal claws of the leg, and from a2 to arista of the antenna (Campbell and Tomlinson, 1998; Cohen and Jurgens, 1989; Dong et al., 2000; Gorfinkiel et al., 1997) (reviewed by Panganiban, 2000). *ss* encodes a bHLH-PAS family transcription factor related to the mammalian dioxin receptor and is required for patterning of the distal part of the first tarsal (t1) and second through fourth tarsal segments (t2-t4) (Duncan et al., 1998). In addition to their proximodistal (PD) patterning roles in the antenna and leg, both *Dll* (Cohen and Jurgens, 1989; Dong et al., 2000; Sato, 1984; Sunkel and Whittle, 1987) and *ss* (Balkaschina, 1929; Burgess and Duncan, 1990; Duncan et al., 1998; Struhl, 1982) are required for antenna specification.

The observations that *bab* is differentially expressed between antenna and leg (Godt et al., 1993) (this work) and that its putative activators, Dll and Ss have distinct expression patterns and functions in the two limb types (Dong et al., 2000; Dong et al., 2001; Duncan et al., 1998), led us to hypothesize that the regulation of *bab* was likely to vary between limbs. Here, we present evidence that supports this. In particular, we find that although Dll does serve as a *bab* activator in the antenna and also in the wing pouch, only part of this activation in the leg and none in the wing is mediated by Ss. Interestingly, the differences between antenna and leg expression of *bab* are not a consequence of differences in activators, but of differences in the expression of *bab* repressors. We conclude that *bab* is differentially regulated in different limbs by tissue-specific combinations of PD patterning genes. The unique gene networks that regulate *bab* in each limb type thereby contribute to morphological variation by regulating the number of distal segments.

MATERIALS AND METHODS

Immunohistochemistry

Antibody stainings and immunohistochemistry were carried out as described previously (Halder et al., 1998). Antibodies used were: rabbit anti-Hth (Pai et al., 1998), rabbit anti-Dll (Panganiban et al., 1995), mouse anti-Dll (Vachon et al., 1992), rat anti-Bab (a gift from F. Laski), mouse anti-Myc (a gift from S. Blair), rabbit anti-β-gal (a gift from S. Carroll), rabbit anti-Sal (a gift from R. Schuh), mouse anti-Dac (University of Iowa Developmental Studies Hybridoma Bank) and rabbit anti-BarH1 (a gift from T. Kojima). Secondary antibodies coupled to Cy2, Cy3 and Cy5 were obtained from Jackson ImmunoResearch. Imaging was carried out on BioRad MRC1024 confocal and Zeiss Axiophot microscopes.

Fly strains

The following fly strains were employed: (1) *dpp-GAL4 (A.3)/TM6B* (Morimura et al., 1996); (2) *act>CD2>GAL4* (=actin promoter-FRT-CD2-FRT-GAL4) (Pignoni and Zipursky, 1997); (3) *w; UAS-GFP-hth8/TM6B, Tb, Hu* (Casares and Mann, 1998); (4) *w; FRT82B* hth^{P2} (Pai et al., 1998); (5) *w;* dac^{4} *FRT40A*, (Graeme Mardon); (6) *w; FRT43D* Dll^{SA1} (Dong et al., 2000); (7) *y hs-FLPase; FRT43D 2piM* (Dong et al., 2000); (8) Dll^{1}*/CyO, wg-lacZ*; (9) Dll^{3}*/CyO, wg-lacZ*; (10) bab^{ARO7}*/TM6B, Tb Hu e ca* (Frank Laski); (11) *Df (3L)* bab^{AR07} *th st cp FRT80B/TM3* (Artyom Kopp); (12) *dac-lacZ* (Graeme Mardon); (13) *w; UAS-Dll/In (2LR) Gla, Gla Bc Elp* (Konrad Basler); (14) *Df(3R)*$ss^{D114.4}$*/TM6* (Ian Duncan); (15) $ss^{D114.7}$*/TM6* (Ian Duncan); and (16) *y hs-FLPase; FRT82B M piM* (S. Blair). Stocks constructed by us for these experiments were: (1) *y hs-FLPase; FRT82B 2piM*; (2) *y hs-FLPase; UAS-GFP-hth8/TM6B, Tb Hu*; (3) *y hs-FLPase; UAS-dac*; (4) Dll^{GAL4}*/CyO, wg-lacZ*; (5) *w; Df(2L)*sal^{5} *FRT40A*; (6) *y hs-FLPase; 2piM FRT40A*; and (7) *y hs-FLPase; UAS-sal*.

Genetic manipulations

Dll hypomorphic larval imaginal discs were generated by crossing heterozygous *Dll* mutant animals in which each *Dll* mutant chromosome was balanced over *CyO, wg-lacZ*. Mutant animals were identified by the absence of X-gal staining in the larval tails. Ectopic expression of *Dll*, *homothorax* (*hth*) and *dachshund* (*dac*) was induced using the GAL4/UAS binary system (Brand and Perrimon, 1993). *dpp-GAL4* was used to activate *UAS-GFP-hth* along the anterior-posterior compartment boundary of the developing imaginal discs. Clones of cells ectopically expressing Hth, Dac or Sal were generated using a modified GAL4/UAS system (Pignoni and Zipursky, 1997) in which *y hs-FLPase; UAS-GFP-hth/TM6B, Tb Hu* (or *y hs-FLPase; UAS-dac* or *y hs-FLPase; UAS-sal*) flies were crossed to *act>CD2>GAL4* flies. The resulting larvae were heatshocked at 37°C for 10 minutes at 72-96 hours after egg laying (AEL) to induce site-specific recombination between the FRT sites, which in turn results in constitutive GAL4 expression in the clones. *Dll*, *hth*, *dac* and *sal* null clones were generated using the FLP/FRT system (Xu and Rubin, 1993). Animals of the genotypes: (1) *y hs-FLPase; FRT43D 2piM/FRT43D* Dll^{SA1}; (2) *y hs-FLPase; FRT82B piM/FRT82B* hth^{P2}; (3) *y hs-FLPase; 2piM FRT40A/*dac^{4} *FRT40A*; and (4) *y hs-FLPase; 2piM FRT40A/Df(2L)*sal^{5} *FRT40A* were heatshocked at 37°C for 1 hour at 48-72 hours AEL and examined in mid- to late-third instar. To make large *hth* null clones, the hth^{+} FRT chromosome carried a *Minute* (*M*) mutation. Animals of the genotype: *y hs-FLPase; FRT82B M piM/FRT82B* hth^{P2} were heatshocked at 37°C for 1 hour at 120-144 hours AEL and examined in mid- to late-third instar (because the heterozygous *Minute* animals develop slowly, this was ~240-288 hours AEL). The *ss* null genotype examined was *Df(3R)*$ss^{D114.4}$/$ss^{D114.9}$. The *bab* null genotype examined was *Df(3L)*bab^{ARO7}/*Df(3L)*bab^{AR07} *th st cp FRT80B/TM3*.

RESULTS

bab expression and function during limb development

bab is required for the proper segmentation of the distal leg and antenna. Segmental fusion is seen in a *bab* null leg and antenna (Fig. 1A-D) (Godt et al., 1993). The two distalmost tarsal joints are most sensitive to loss of *bab*, but fusion of all tarsal segments is seen in *bab* null animals. This also results in

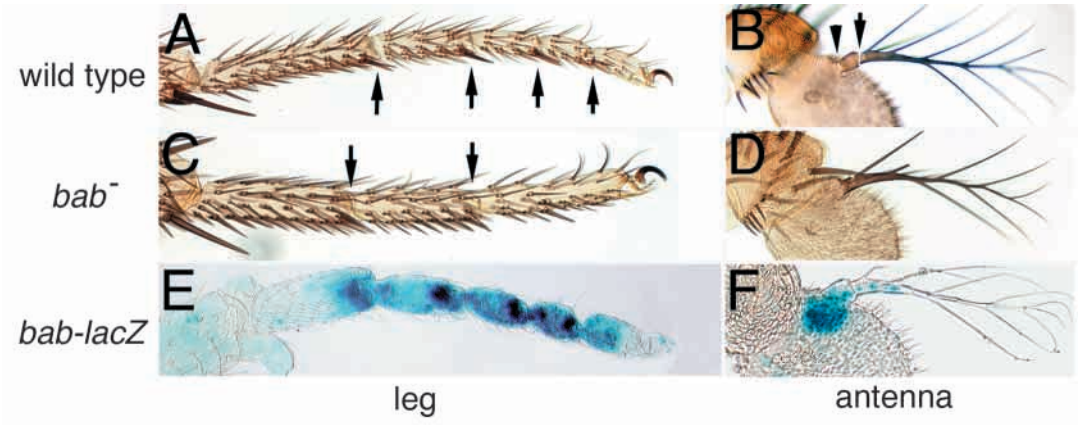

Fig. 1. *bab* is required for joint formation in the distal leg and antenna. Wild-type cuticles of *Drosophila* leg (A), and antenna (B). Tarsal joints in the leg are indicated by arrows. The a4/a5 joint in the antenna is indicated by an arrowhead, the a5/arista joint by an arrow. (C) Tarsal joints (arrows) are fused or absent in the *bab* null leg (Godt et al., 1993). (D) The a4/a5 and a5/arista joints are absent in the *bab* null antenna (Godt et al., 1993). (E,F) The expression patterns of *bab-lacZ* during pupal stages are consistent with the phenotypes of the adult tarsal and antennal joints.

shortening the overall length of the tarsus (Godt et al., 1993). In the antenna, because of loss of the a4/a5 and a5/arista joints, the arista is fused to a4. Consistent with its function, *bab* is expressed at the distal end of each tarsal segment during pupal leg development and is expressed in distal a3 and in the a4 and a5 segments of the antenna (Fig. 1E,F).

Dynamic expression of *bab* during antenna and leg development

In order to gain insight into possible positive and negative regulators of *bab* expression during appendage development, we first examined the dynamics of wild-type *bab* expression. In late second/early third instar antenna and leg discs, *bab* is expressed throughout most of the *Dll* domain, with the highest levels of expression in the centers of both antennal and leg discs (Fig. 2A,A′,D,D′). By mid-third instar, *bab* expression is lost from the distalmost cells, and a ring of Bab is apparent in the antennal and leg discs (Fig. 2B,B′,E,E′). Still later in the third instar, the *bab* expression pattern resolves into a set of concentric rings, two in the antenna and four in the leg (Fig.

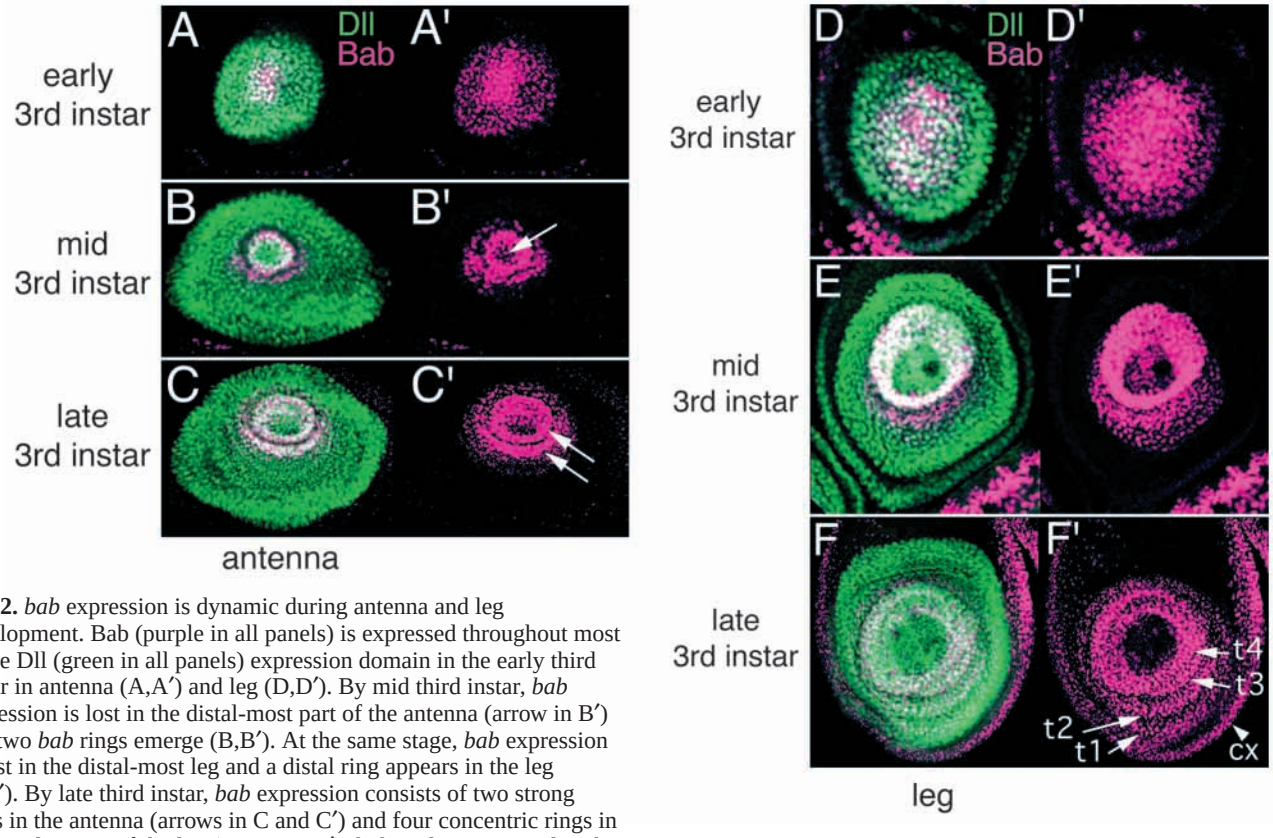

Fig. 2. *bab* expression is dynamic during antenna and leg development. Bab (purple in all panels) is expressed throughout most of the Dll (green in all panels) expression domain in the early third instar in antenna (A,A′) and leg (D,D′). By mid third instar, *bab* expression is lost in the distal-most part of the antenna (arrow in B′) and two *bab* rings emerge (B,B′). At the same stage, *bab* expression is lost in the distal-most leg and a distal ring appears in the leg (E,E′). By late third instar, *bab* expression consists of two strong rings in the antenna (arrows in C and C′) and four concentric rings in the tarsal region of the leg (arrows in F′). *bab* is also expressed in the presumptive coxa (cx) where it does not overlap with Dll.

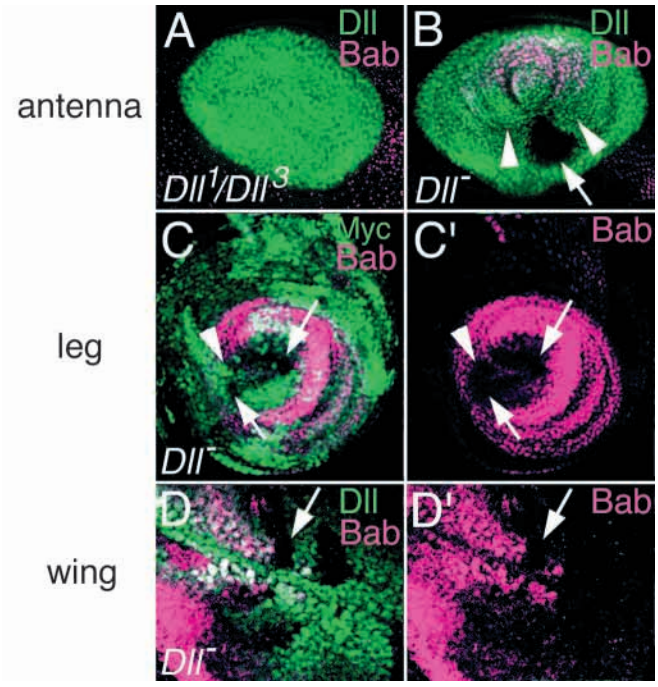

Fig. 3. *bab* expression depends on Dll in the antenna, leg and wing. Bab (purple in all panels) is not detected distally in Dll^{1}/Dll^{3} hypomorphic antennal discs (A). Note that some Dll (green) is still present in the hypomorphic discs. Bab is not detected in (arrow) or around (arrowheads) a *Dll* null clone in the antenna (B). Double labeling of similar antennal clones for Dll and a *myc* marker indicates that Dll is lost cell-autonomously, i.e. loss of Dll expression corresponds with loss of Myc expression. The non cell-autonomous loss of Bab surrounding these *Dll* null antennal clones is correlated with activation of Dac (Dong et al., 2001), which we demonstrate here is a *bab* repressor. Bab also is not detected within (arrows) or around *Dll* null clones in the leg (C,C′). These clones were identified by absence of expression of the *myc* marker. The arrowhead indicates a portion of a Dll^{+} myc^{+}/Dll^{+} myc^{+} "twin spot" in which Bab expression has been lost non cell-autonomously. The twinspots carry twice as many copies of the *myc* gene than the surrounding Dll^{+} myc^{+}/Dll^{-} heterozygous tissue and therefore stain more brightly. Although Bab expression is lost non cell-autonomously around *Dll* null clones in both antenna and leg discs, the mechanism by which this occurs in each may differ since Dll expression is also lost non cell-autonomously around the *Dll* null clones in the leg (J. C. and G. P., unpublished). Bab is lost cell-autonomously from a *Dll* null clone in the wing (arrow in D and D′).

2C,C′,F,F′). Based on its wild-type expression, we postulated that *bab* is activated initially throughout the *Dll* domain, and that the mature *bab* expression patterns are achieved in part by subsequent repression distally and proximally. In addition, *bab* either is partially repressed or its expression is not maintained between concentric rings.

bab expression depends on Dll in the antenna, leg and wing

To examine the relationship between *bab* and Dll in the antenna, leg and wing, we investigated *bab* expression in *Dll* hypomorphic discs and in clones of cells lacking Dll. Bab is not detected in either *Dll* hypomorphic antenna (Fig. 3A) or leg (Campbell and Tomlinson, 1998) (not shown) discs, or in

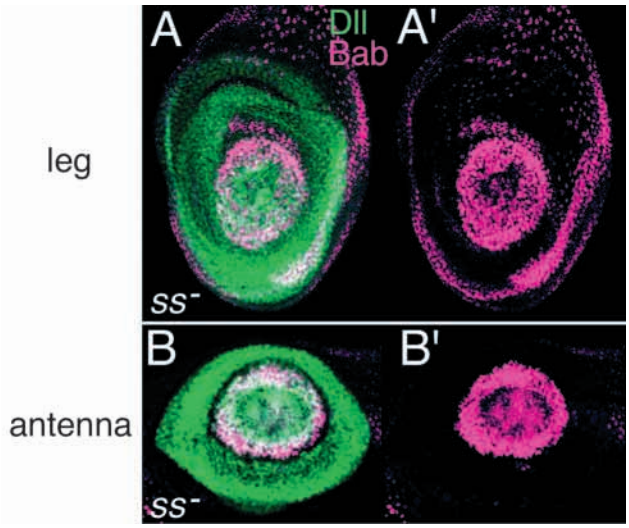

Fig. 4. *bab* expression partially depends on Ss in the antenna and leg. (A,A′) Proximal tarsal rings of Bab (purple in all panels) expression are lost in the *ss* null leg, while a distal ring of *bab* expression remains. (B,B′) Expression of *bab* in the coxa is unaffected in *ss* mutants. Bab expression is partially lost in the *ss* null antenna, only one ring remains. Since these antennae are transformed toward leg, this ring is likely to represent the distal leg ring of *bab*. *bab* expression is partially derepressed distally in both the *ss* mutant leg and antenna. Expression of Dll appears normal in these discs (green in A and B).

Dll null clones in either the antenna or leg (Fig. 3B,C,C′). Interestingly, although *bab* expression is lost cell-autonomously in Myc-marked *Dll* null clones in both antenna and leg (arrows in Fig. 3B,C,C′ and not shown), *bab* expression is also lost non cell-autonomously under certain conditions. In the antenna, for instance, *bab* expression is lost in cells surrounding the *Dll* null clones when those clones are near the boundary of the normal *dac* and *bab* expression domains (arrowheads in Fig. 3B). Non cell-autonomous loss of the modulation between *bab* rings is also seen occasionally near these *Dll* null clones. These phenomena likely are due to the non cell-autonomous activation of *dachshund* (*dac*) around the *Dll* null clones (Dong et al., 2001), since Dac is a *bab* repressor (see below and Discussion). In the leg, we also observe non cell-autonomous loss of *bab* expression around *Dll* null clones (e.g. arrowhead in Fig. 3C,C′). However, in this case *Dll* expression is also lost (J. C. and G. P., unpublished data). Thus the non cell-autonomous loss of *bab* expression in the leg is likely due to the loss of the *bab* activator Dll, and mechanistically different from the non cell-autonomous loss of *bab* expression in the antenna.

bab expression partially overlaps with Dll in the wing pouch. Although *bab* expression is not visibly reduced in *Dll* hypomorphic wings (not shown), *bab* expression is lost in some *Dll* null clones in the wing pouch (arrow in Fig. 3D,D′). There is no detectable adult phenotype in *bab* null wings (not shown). We conclude that *bab* activation requires *Dll* in the antenna, in the leg and in part of the wing pouch.

bab expression partially depends on Ss in the antenna and leg

ss null mutations result in loss of part of t1 and of the t2, t3

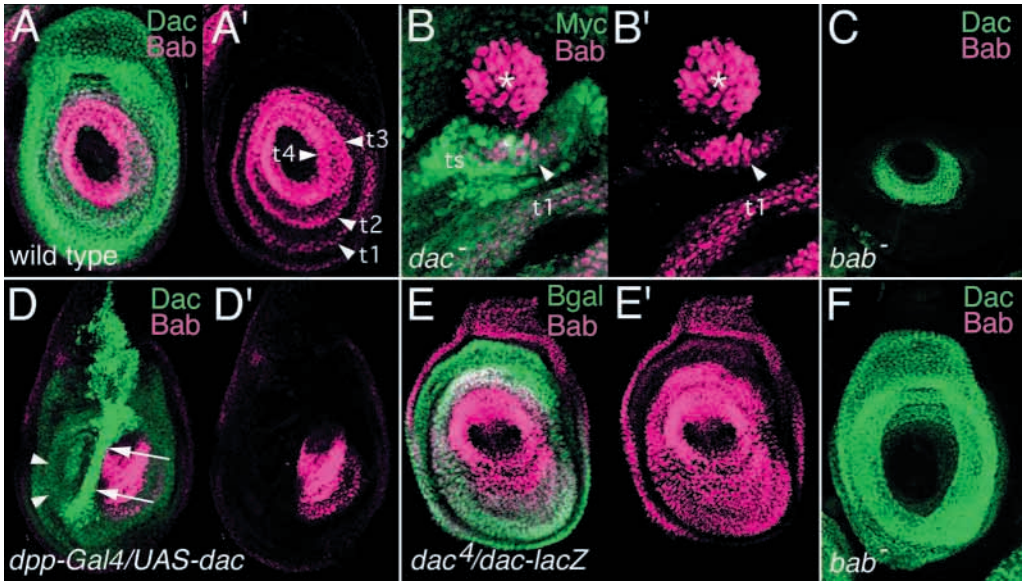

Fig. 5. Dac restricts *bab* expression in the leg. (A,A′) Wild-type expression of Bab (purple in all panels) and Dac (green) in the leg disc. (B,B′) *bab* expression is activated in a *dac* null clone (asterisk). The clone is marked by the absence of Myc (green). The wild-type twin spot (ts) of the clone possesses two copies of the *myc* transgene and therefore appears brighter green. Note that *bab* is non cell-autonomously activated in part of the twin spot near the clone (arrowhead). A portion of the endogenous *bab* ring of the first tarsal segment is indicated (t1). (D,D′) *bab* expression is repressed by ectopic Dac (arrows) produced in the *dpp* pattern. The *dpp* pattern is dynamic (Masucci et al., 1990; Weigmann and Cohen, 1999), and Bab is not detected in cells that were exposed to high levels of Dac earlier in development and that continue to express moderate levels of Dac, probably due to autoregulation (arrowheads). (E,E′) Bab expression expands proximally and its modulation between rings is greatly diminished in *dac* mutant leg discs. Dac appears to be expressed normally in *bab* null antennal (C) and leg (F) discs.

and t4 segments of the leg, including their joints. Thus in *ss* null legs, there remains only a single distal joint positioned between t1 and t5 (Duncan et al., 1998). Consistent with the adult cuticular phenotype, in late third instar *ss* null animals, we observe a single ring of *bab* expression in the leg (Fig. 4A,A′). Double labeling of the *ss* mutant leg discs with both Bar antibodies that label the t4 and t5 segments (Kojima et al., 2000) and Bab antibodies (not shown), indicates that the remaining Bab represents the distal-most ring. Thus while the proximal tarsal rings of *bab* depend on Ss, the distal ring expression of *bab* is Ss-independent. Similarly, in *ss* null antennal discs, there is a distal ring of *bab* expression (Fig. 4B,B′). This is consistent with the presence of a single distal joint, between the transformed a3 and the transformed arista of *ss* null animals (Duncan et al., 1998). However, because the *ss* null antenna exhibit transformations toward leg, it is difficult to evaluate the requirements for *ss* in normal antennal joint formation. Thus, while we can conclude that Ss mediates only part of the Dll activation of *bab* in the leg, the relationship between *ss* and *bab* during normal antennal development remains unknown.

The proximal limit of *bab* expression in the leg is determined by Dac repression

Although *bab* is expressed throughout most of the *Dll* domain at early third instar, by late third instar, *bab* becomes restricted to a portion of the *Dll* domain. We therefore hypothesized that there are *bab* repressors that are differentially expressed in the complementary part of the *Dll* domain. One candidate *bab* repressor in the leg is Dac. Dac is a novel nuclear protein required for patterning of the trochanter, tibia and proximal tarsal segments (Mardon et al., 1994). The distal boundary of *dac* expression is dynamic, encompassing increasingly more distal segments as the leg disc grows (Lecuit and Cohen, 1997). When *dac* is first activated in the leg at late second instar, its expression overlaps only slightly with that of *Dll*. However, by late third instar, *dac* expression also includes the tibia and the first and second tarsal segments (t1 and t2) of the leg disc, where it overlaps with *Dll* (Lecuit and Cohen, 1997) and with the t1 and t2 *bab* rings (Fig. 5A,A′). To test whether Dac is a repressor of *bab* in the leg, we carried out both loss- and gain-of-function experiments. We observe strong activation of *bab* in *dac* null clones (asterisk in Fig. 5B,B′) and repression of *bab* by ectopic Dac (Fig. 5D,D′). Therefore, Dac is a repressor of *bab* in the leg. We note that *bab* can be non cell-autonomously activated near *dac* null clones (arrowhead in Fig. 5B,B′), although we do not yet know the mechanism by which this occurs. *Dll* also is activated in the *dac* null clones that exhibit strong *bab* expression and *Dll* is repressed by ectopic Dac

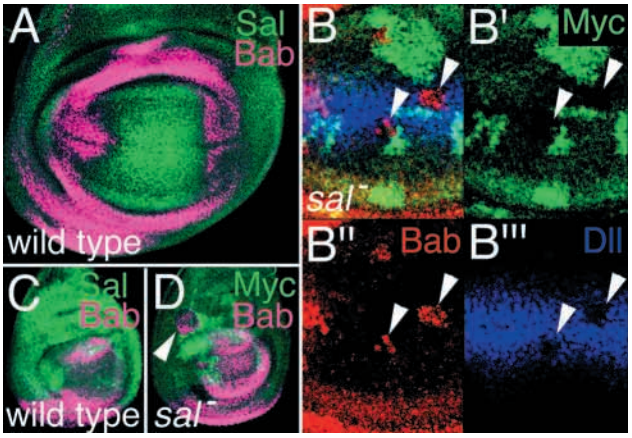

Fig. 6. Sal restricts *bab* expression in the wing and haltere. (A) Wild-type expression of Bab (purple) and Sal (green) in the wing disc. (B,B′,B″,B‴) *sal* null clones (marked by the absence of green Myc staining) in the center of the wing pouch (arrowheads) lose Dll (blue) expression and activate Bab (red). (C) Wild-type expression of Sal (green) and Bab (purple) in the haltere. (D) A *sal* null clone in the haltere disc (arrowhead) also activates Bab.

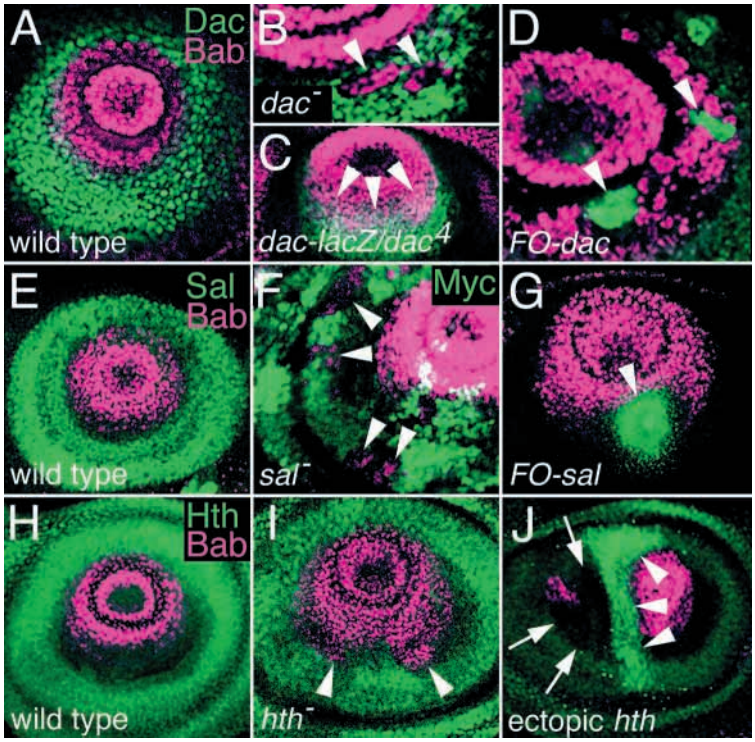

Fig. 7. *bab* expression is repressed by Dac, Sal and Hth in the antenna. (A) Wild-type expression of Bab (purple in all panels) and Dac (green in A,B,D). (B) Bab is derepressed in *dac* null clones in a3 (arrowheads). (C) *bab* expression expands proximally (arrowheads) into a3 in *dac-lacZ/dac*^{4} discs. Green is β-galactosidase produced by the *dac-lacZ* element and marks where Dac would have been expressed. Note the extensive overlap (white) in C not seen in A. (D) *bab* is repressed by ectopic Dac produced in flipout clones (arrowheads). (E) Wild-type expression patterns of Bab and Sal (green). Sal-expressing cells were visualized by use of an anti-β-galactosidase antibody in a *sal-lacZ* background. (F) *bab* is derepressed in *sal* null clones in a2 (arrowheads). Clones are marked by the absence of Myc (green). (G) *bab* is repressed by ectopic Sal (green) produced in a flipout clone (arrowheads). (H) Wild-type expression of Bab and Hth (green in H-J). (I) *bab* is activated in *hth* null clones (arrowheads). (J) *bab* is repressed by ectopic Hth (arrowheads) produced in the *dpp* pattern. The *dpp* pattern is dynamic (Masucci et al., 1990; Weigmann and Cohen, 1999), and Bab is not detected in cells exposed to high levels of Hth earlier in development (arrows in J).

(Abu-Shaar and Mann, 1998; Dong et al., 2001). Thus Dac repression of *bab* in the leg could be mediated in part by reducing Dll activity. Nonetheless, the fact that *Dll*, *dac* and *bab* are coexpressed in t1 and t2 by late third instar, indicates that Dac is insufficient to repress *Dll* or *bab*.

In addition to setting the proximal boundary of the *bab* domain in the leg, Dac also plays a role in modulating *bab* expression between rings. In strong *dac* hypomorphic leg discs, Bab loses its inter-ring modulation and is uniformly expressed at high levels in t1-t3 (Fig. 5E,E′). Interestingly, the uniform Bab expression in *dac* null legs is correlated with phenotypes similar to *bab* loss of function, namely the t1-t3 tarsal segments and joints are lost (P. D. S. D. and G. P., unpublished results). Thus, alternating high and low levels of Bab appear to be critical for tarsal joint formation.

Bab does not set the distal limit of *dac* expression in either the antenna or the leg

It has been proposed that mutually repressive interactions in the leg, e.g between *Bar* and *aristaless* (*al*) (Kojima et al., 2000) and between *Dll* and *dac* (Dong et al., 2001), play critical roles in the subdivision of the PD axis. We therefore wished to test whether the antagonism of Dac for Bab is reciprocal. In *bab* null antenna and leg discs, Bab protein cannot be detected, but *dac* expression is normal (Fig. 5C and F). Consistent with this, *dac* is expressed in a medial ring instead of a circle that encompasses the *bab* domain prior to *bab* activation (not shown). Thus Dac represses *bab*, but Bab does not repress *dac*.

Restriction of *bab* expression by Spalt in the wing

We have also investigated *bab* regulation in the developing wing. In the wing pouch, *bab* expression resembles that of *Dll*, except that *bab* is not expressed in the middle of the wing pouch along the anterior-posterior compartment boundary. This absence of detectable *bab* expression coincides with the domain of *spalt* (*sal*) expression (Fig. 6A). *sal* encodes a zinc finger transcription factor (Kuhnlein et al., 1994) required to position the L2 wing vein (de Celis et al., 1996; Sturtevant et al., 1997). To test whether Sal represses *bab*, we made *Df(2L)sal*^{5} clones. We found that *bab* is derepressed in these *sal* null clones (arrowheads in Fig. 6B,B′,B″). Therefore, *bab* expression is restricted by Sal in the wing. *bab* activation in the *sal* null clones is unlikely to be mediated via Dll, since Dll is not expressed or activated in many of these clones (arrowheads in Fig. 6B‴). *bab* is activated similarly in *sal* null clones in the haltere (Fig. 6C,D) and this is not mediated by Dll either (not shown). In fact, the wild-type expression of *bab* does not overlap with *Dll* in the haltere (not shown). Together, our results indicate that some, but not all, *bab* activation in the wing is mediated by Dll and that Sal is a potent repressor of *bab* in both wing and haltere.

The proximal limit of *bab* expression in the antenna is determined by Dac, Sal and Hth repression

As demonstrated above, Dac represses *bab* in the leg, and Sal represses *bab* in the wing pouch. The expression of both Dac and Sal are more or less complementary to that of *bab* in the antenna, as is that of Hth (Fig. 7A,E,H). Thus all three genes are potential *bab* repressors in the antenna. Hth is a TALE homeodomain transcription factor required for the nuclear localization of Extradenticle (Exd), a Pbx-related homeodomain protein (Abu-Shaar et al., 1999; Kurant et al., 1998; Pai et al., 1998; Rieckhof et al., 1997). Both Hth and Exd are required for patterning of the coxa and trochanter of the leg and of the first and second antennal segments (Abu-Shaar and Mann, 1998; Aspland and White, 1997; Gonzalez-Crespo and Morata, 1995; Gonzalez-Crespo and Morata, 1996;

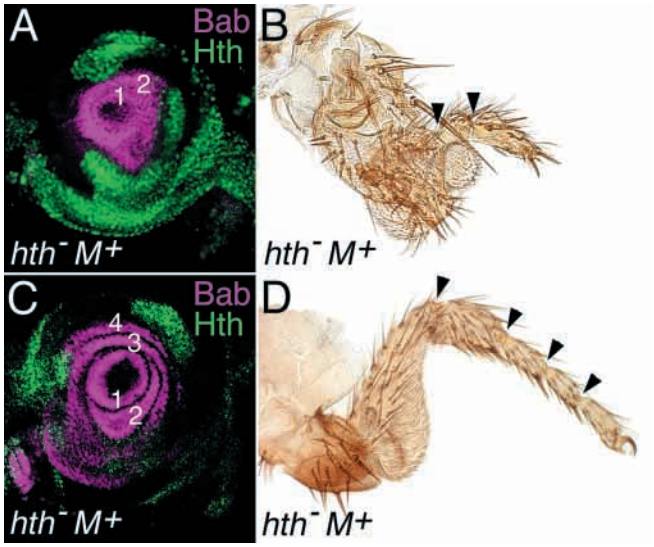

Fig. 8. Variations in *bab* derepression in Hth null antennae are correlated with variations in the resulting numbers of tarsal joints. Large *hth* null clones in the antenna were generated using a *Minute* (*M*) mutation on the hth^+ chromosome. The $hth^+ M^-/hth^+ M^-$ twinspots die, while the $hth^+ M^-/hth^- M^+$ heterozygous tissue surrounding the *hth* null clones grows poorly. Thus much of the antennal disc comprises $hth^- M^+$ tissue and is transformed toward leg. Under these conditions, Bab (purple) is often derepressed uniformly (A), and sometimes derepressed in a modulated pattern (C) resembling that found in the leg. (B) A partially transformed antenna with two joints (arrowheads). (D) A more completely transformed antenna with five tarsal segments and four tarsal joints (arrowheads). The relative frequencies at which these cuticular phenotypes arise suggest that the cuticle in B may derive from a disc with Bab expression similar to that found in A, while the cuticle in D may derive from a disc with Bab expression similar to that found in C. See text for details.

Pai et al., 1998; Rauskolb et al., 1993; Rauskolb et al., 1995; Rieckhof et al., 1997). In addition, Hth and Exd are required cell-autonomously throughout the antenna to specify antennal identity (Casares and Mann, 2000; Gonzalez-Crespo and Morata, 1995; Pai et al., 1998). The antennal expression of both *dac* and *sal* is dependent on Hth and Exd (Dong et al., 2000; Dong et al., 2001). Dac is coexpressed with Hth, nuclear Exd (n-Exd) and Sal in a3 (Fig. 7A and not shown), while Sal is coexpressed strongly with Hth and n-Exd in a2 (Fig. 7E and not shown). The expression of Sal in a3 is strong at early third instar and weak at late third instar. Thus the combination of Hth, n-Exd, Dac and Sal marks the proximal limit of *bab* expression. To test whether these genes repress *bab* to set its proximal boundary, we carried out both loss- and gain-of-function experiments.

bab is derepressed in distal a3 in *dac* null clones (Fig. 7B), and the *bab* domain expands proximally in strong *dac* hypomorphs (Fig. 7C). *dac* null clones and *dac* null antennal discs do not exhibit derepression of *bab* in a1 or a2 (Fig. 7C and not shown). Further, *bab* is repressed in flipout clones ectopically expressing Dac (Fig. 7D). Based on these results, we conclude that Dac is a *bab* repressor in distal a3. As in the leg, loss of inter-ring modulation of *bab* is observed in the antenna in strong *dac* hypomorphs (Fig. 7C) and this loss is correlated with loss of the a4/a5 and a5/arista joints (P. D. S. D., J. S. Dicks and G. P., unpublished data). Thus modulation of *bab* expression appears to be necessary for distal antennal as well as distal leg joint formation.

bab is derepressed weakly in *sal* null clones in a2 (Fig. 7F) and repressed in flipout clones ectopically expressing Sal (Fig. 7G). *sal* null clones do not exhibit derepression of *bab* outside of a2 (Fig. 7F and not shown). We conclude that Sal is a *bab* repressor in a2. *bab* often is derepressed in *hth* null clones in both a2 and a3 (Fig. 7I). We have shown previously that while *sal* is lost in *hth* null clones (Dong et al., 2000), *dac* is derepressed in *hth* null clones in both a2 and a3 (Dong et al., 2001). Thus *hth* null clones in a2 and a3 often coexpress *bab* and its repressor *dac*. This supports the leg data described above indicating that Dac is insufficient for *bab* repression. We conclude that Dac probably requires Hth (or the product of a gene activated by Hth) as a corepressor in the antenna.

Interestingly, in large *hth* null clones that encompass much of the distal antenna, *bab* often (40/66 discs=61%) is derepressed uniformly throughout a2 and a3 (Fig. 8A), while at other times (26/66 discs=39%) the derepressed *bab* is modulated and appears in rings. Among the *hth* mutant antennal discs in which the derepressed *bab* is modulated, we frequently (11/26 discs=42%) see four rings (Fig. 8C). Thus large clones with four rings constitute 17% (11/66) of the total large *hth* null clones examined. This frequency resembles that (16%; 10/61) at which five distinct tarsal segments separated by four joints can be detected in adult antennae harboring large *hth* null clones (Fig. 8D). In these experiments, the remaining 51/61 antennae, while visibly transformed toward leg, possessed three or fewer distinct tarsal-like joints and four or fewer tarsal segments (Fig. 8B). Thus inter-ring modulation in *hth* mutant antennae can be correlated with joint formation, and *bab* modulation probably is essential for normal distal joint formation in both the antenna and the leg.

Ectopically expressed Hth can repress *bab* in the antenna (Fig. 7J), but expression of *dac* (Dong et al., 2001) and *sal* (Dong et al., 2000) is also activated in these cells. We therefore cannot distinguish whether ectopic Hth is sufficient for *bab* repression or whether Hth cooperates with Dac and Sal to repress *bab*. Nonetheless, the loss- and gain-of-function data presented here indicate that all three factors function in limiting *bab* expression in the antenna. Sal and Hth represses *bab* in a2, while Dac and Hth repress *bab* in a3.

DISCUSSION

Differential regulation of *bab* and limb morphology

The precision of pattern formation in development is the result of fine control and balance of dynamic webs of gene regulation. Here we have investigated a portion of the patterning processes in the appendages of *Drosophila*. The differences in how *bab* is regulated in different limb types and within a single limb type along the PD axis indicate that there are multiple ways to alter *bab* expression by modulating activator and/or repressor activity. Because *bab* is required for formation of distal joints, these possibilities are likely to facilitate variations in where joints form and how many segments a limb possesses, thereby contributing to the morphological diversity of legs and antennae.

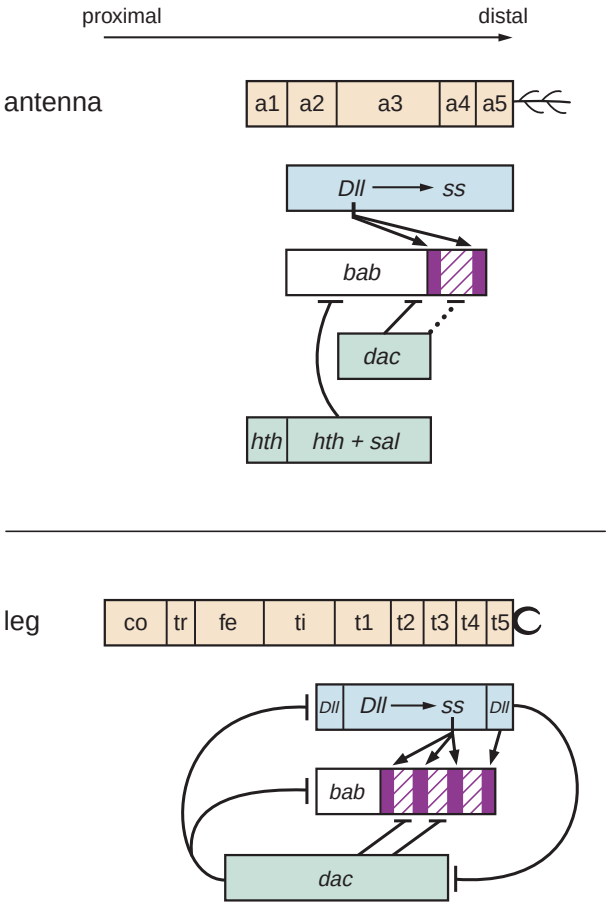

Fig. 9. Both conserved and unique interactions regulate *bab* along the PD axis of the antenna and leg. *bab* is activated by *Dll* in both antenna and leg primordia. *bab* expression is *ss* dependent in the first through third tarsal segments (t1-3), but not in the fourth tarsal segment (t4) of the leg. The requirement of *ss* for *bab* expression in the antenna could not be assessed, because *ss* null antennae are transformed toward leg. The proximal boundary of *bab* expression is set by *dac* in both antenna and leg, but *hth* and *sal* also are required for *bab* repression in the antenna. *dac* also modulates *bab* expression levels between rings in both antenna and leg. The dependence of *ss* expression on *Dll* has been documented previously (Duncan et al., 1998), as have the dependence of *sal* (Dong et al., 2000) and *dac* (Dong et al., 2001) expression on *Dll* and *hth* and the mutual antagonism between *Dll* and *dac* in the leg (Dong et al., 2001). Solid lines indicate cell-autonomous effects that could be direct. The dotted line indicates that there is a non cell-autonomous component to inter-ring modulation of *bab* by *dac* in the antenna. See text for details.

Activation of *bab* by Dll and Ss

Both *ss* and *bab* expression depend on Dll in the antenna and leg (Campbell and Tomlinson, 1998; Duncan et al., 1998) and this work). We report here that only a subset of the Dll-dependent *bab* expression depends on Ss. In particular, the tarsal rings of *bab* expression in distal t1, distal t2 and distal t3 are both Dll and Ss dependent, whereas the ring of *bab* in distal t4 depends on Dll but not on Ss. Thus for the t1, t2 and t3 rings, Ss either mediates the Dll activation of *bab* or cooperates with Dll to activate *bab*, while the t4 ring is activated independent of Ss, possibly directly by Dll (Fig. 9). *bab* expression in the t4 ring may be regulated via a different enhancer than *bab* expression in the t1, t2 and t3 rings, but whether or not there are distinct *bab* enhancers for different rings is unclear. We think it likely that *bab* expression in each of the four tarsal rings represents differential activation in response graded Dpp and Wg signals in conjunction with the Dll and/or Ss selector(s).

In the antenna, the effects of Ss on *bab* are difficult to interpret because *ss* null antennae exhibit transformations toward leg. In these transformed discs, the *bab* expression pattern resembles that of *ss* null leg discs, namely only a single distal ring is observed. This may represent the distal leg ring. However, since *bab* enhancer elements have not yet been identified, we cannot determine whether the distal ring in both antenna and leg represents activation via a shared enhancer or utilizes distinct enhancers in each limb type.

We find that only part of the *bab* expression depends on Dll in the wing, and that in a homologous structure, the haltere, *bab* is not Dll-dependent. It has been suggested that the insect wing may derive from a proximodorsal part of a branched limb of their aquatic ancestors (Averof and Cohen, 1997; Kukalova-Peck, 1978). There is expression of *bab* proximal to the *Dll* domain in both antenna and leg discs. Thus if the wing has derived from the proximal leg, it could be that the regulation of *bab* expression in the wing shares features with that of the proximal leg.

Differential restriction of *bab* in different appendage types by Dac, Sal and Hth

Here we have demonstrated that repression by Dac establishes the proximal border of *bab* expression in the leg, that Sal restricts *bab* expression in the wing, and that Dac, Sal and Hth establish the proximal limit of *bab* expression in the antenna (Fig. 9). We have shown previously that coexpression of Dll and Hth/Exd determines antennal identity, and activates *sal* (Dong et al., 2000) and *dac* (Dong et al., 2001). Here we demonstrate that differential expression of these genes leads to significant differences in *bab* expression. In particular, *bab* expression is restricted to a small domain containing two rings in the antenna in contrast to a larger domain of four rings in the leg. As *bab* is necessary for joint formation, we propose that these differences in *bab* expression contribute to the differences in the number of distal segments found in the antenna and the leg.

In both antenna and leg discs, *bab* expression is derepressed in *dac* null clones that are proximal to normal *bab* expression domain. However, the mechanisms of *bab* derepression probably differ between limb types. In the leg, derepression of *bab* is associated with derepression of *Dll*. Therefore, Dac likely restricts *bab* expression via *bab* activators. In the antenna, *Dll* and *dac* expression normally overlap, and *Dll* continues to be expressed in *dac* null clones. This suggests a more direct repression mechanism may exist in the antenna. There is substantial overlap between *dac* and *bab* expression in the leg at late third instar, indicating that the presence of Dac protein is not sufficient for *bab* repression and that the presence or absence other factors is required in order for Dac to repress *bab*. This is perhaps not surprising since Dac does not contain a known DNA binding motif and forms complexes with Eyes absent and Sine oculis to regulate transcription during eye development (Chen et al., 1997; Pignoni et al., 1997). Based

on existing genetic information, candidate Dac corepressors are Sal, Hth and n-Exd in the antenna, and Antennapedia (Antp) in the leg. Alternatively, because Dac overlaps with Dll in both the antenna and leg where Dac serves as a *bab* repressor, it also is possible that Dac functions by precluding the ability of Dll and/or Ss to activate *bab*.

Modulation between rings

At early third instar, *bab* is expressed throughout the Dll domain in the leg, with uniformly strong expression in the presumptive tarsal segments and weaker expression in presumptive femur and tibia. By late third instar, *bab* expression is modulated in the presumptive tarsus and is strongest at the distal part of tarsal segments 1-4, immediately proximal to each of the tarsal joints. Weaker expression of *bab* is observed between these concentric rings. This modulation persists at least through early pupal stages. Various mechanisms by which this modulation is achieved can be imagined, including both active repression and/or the failure to maintain *bab* in the inter-rings.

We have indirect evidence suggesting that active repression plays a role. Namely, in *dac* null antenna and leg discs, there is loss of *bab* inter-ring modulation as well as proximal derepression. This lack of inter-ring modulation correlates with joint loss and segment fusion in the *dac* null animals (Dong et al., 2001) (this work). In the leg, *bab* modulation by Dac occurs cell-autonomously. However in the antenna, there is loss of *bab* modulation even in cells that do not normally express detectable levels of Dac. Therefore in addition to repressing *bab* cell-autonomously, Dac also may non cell-autonomously regulate the production of *bab* inter-ring repressor(s) (Fig. 9). Since Dac is a nuclear protein, it could be that Dac directs the expression of a secreted molecule that in turn mediates inter-ring modulation.

Regulation of *bab* and limb evolution

We have presented evidence that *bab* is differentially regulated in different *Drosophila* appendage types and that this regulation contributes to variations in their morphology. It is necessary to determine whether *bab* is required for limb segmentation in other animals, and, if so, whether variations in *bab* regulation contribute to the interspecific variations of homologous appendages. Investigation of the genetic hierarchies that pattern the appendages of other arthropods and of closely related phyla are needed to address these issues and to provide further insights into the generation of morphological diversity.

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