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Essential roles for *lines* in mediating leg and antennal proximodistal patterning and generating a stable Notch signaling interface at segment borders

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ABSTRACT

The *Drosophila* leg imaginal disc provides a paradigm with which to understand the fundamental developmental mechanisms that generate an intricate appendage structure. Leg formation depends on the subdivision of the leg proximodistal (PD) axis into broad domains by the leg gap genes. The leg gap genes act combinatorially to initiate the expression of the Notch ligands *Delta* (*Dl*) and *Serrate* (*Ser*) in a segmental pattern. *Dl* and *Ser* induce the expression of a set of transcriptional regulators along the segment border, which mediate leg segment growth and joint morphogenesis. Here we show that Lines accumulates in nuclei in the presumptive tarsus and the inter-joints of proximal leg segments and governs the formation of these structures by destabilizing the nuclear protein Bowl. Across the presumptive tarsus, *lines* modulates the opposing expression landscapes of the leg gap gene *dachshund* (*dac*) and the tarsal PD genes, *bric-a-brac 2* (*bab*), *apterous* (*ap*) and *BarH1* (*Bar*). In this manner, *lines* inhibits proximal tarsal fates and promotes medial and distal tarsal fates. Across proximal leg segments, *lines* antagonizes *bowl* to promote *Dl* expression by relief-of-repression. In turn, *Dl* signals asymmetrically to stabilize Bowl in adjacent distal cells. Bowl, then, acts cell-autonomously, together with one or more redundant factors, to repress *Dl* expression. Together, *lines* and *bowl* act as a binary switch to generate a stable Notch signaling interface between *Dl*-expressing cells and adjacent distal cell. *lines* plays analogous roles in developing antennae, which are serially homologous to legs, suggesting evolutionarily conserved roles for *lines* in ventral appendage formation.
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Introduction

The *Drosophila* leg imaginal disc provides a tractable system with which to investigate the molecular mechanisms and regulatory logic of limb development (Galindo and Couso, 2000; Kojima, 2004). The leg primordium originates in the embryonic surface ectoderm as a cluster of approximately 20–30 cells, which subsequently invaginates to form a flattened epithelial disc. During larval development disc cells proliferate rapidly to generate a concentrically folded epithelial layer composed of approximately 20,000 cells. During these stages the disc is progressively subdivided into six "true" segments independently movable by muscle: the coxa (co), trochanter (tr), femur (fe), tibia (ti), tarsus (tr), and pretarsus (pt). The tarsus is further subdivided into five nonmusculated subsegments (Fig. 1A) (Cohen, 1993; Fristrom and Fristrom, 1993).

Leg development depends on the subdivision of the anteroposterior (AP), dorsoventral (DV) and proximodistal (PD) axes of the leg primordium into progressively smaller domains. Many of the genes and pathways that establish these axes have been identified. However, it remains unclear how new PD domains are added to the growing leg during development, how the leg PD axis is progressively subdivided into a series of segments, and how segments acquire their unique size and morphological features.

The early limb field is established during embryogenesis in the surface ectoderm and is subdivided into a proximal domain expressing *homothorax* (*hth*) and a distal domain expressing *Dll* (Cohen et al., 1989; Abu-Shaar and Mann 1998; Wu and Cohen 1999). *hth* and *Dll* code for conserved homeobox proteins. The further elaboration of the PD axis is mediate by the morphogens Decapentaplegic (Dpp) and Wingless (Wg) that emanate from dorsal and ventral sources along the AP compartment boundary (Basler and Struhl, 1994; Campbell et al., 1993; Diaz-Benjumea et al., 1994). Wg and Dpp cooperate to induce *dac* expression between the *Dll* and *hth* domains at an intermediate PD position (Lecuit and Cohen, 1997; Abu-Shaar and Mann 1998). *dac* codes for a pioneer nuclear protein (Mardon et al., 1994). Additionally, *wg* and *dpp* cooperate to establish a secondary pattern-organizing center at the distal tip of the leg. Ligands that emanate from this organizer activate the Epidermal Growth Factor receptor (EGF receptor) pathway to control the expression of the tarsal PD genes in a graded manner (Campbell, 2002; Galindo et al., 2002). While *hth*, *dac* and *Dll* respectively control the formation of broad proximal,

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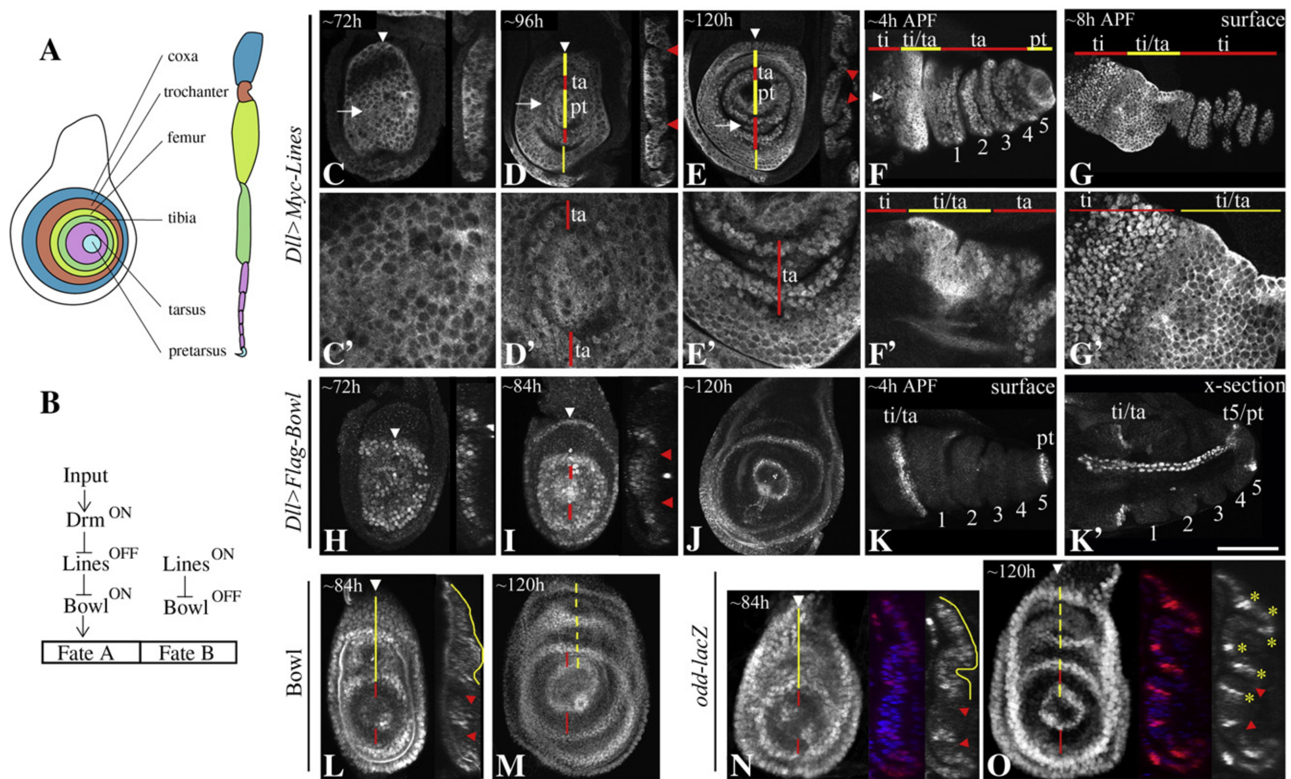

Fig. 1. The dynamics of Lines and Bowl distribution during leg disc development. (A) A cartoon depicting the PD subdivisions of a mature leg disc and an adult leg. (B) The interactions linking Drm, Lines and Bowl in embryonic patterning. (C–G') *Dll>Myc-Lines*; red bars and arrowheads indicate regions where Myc-Lines was nuclear, yellow bars areas where Lines was cytoplasmic. (H–L) *Dll>Flag-Bowl;* Red bars and arrowheads point to regions where Flag-Bowl was downregulated. White arrowheads in C–F, H–I, L and O point to plane of Z-section shown in corresponding insets. White arrows in panels C–E point to magnified regions shown in corresponding lower panels. (C, C') ~72 h; Myc-Lines was broadly cytoplasmic. (D, D') ~96 h; Myc-Lines accumulated in nuclei in the central fold in the emerging tarsus (ta). (E) ~120; Myc-Lines was nuclear in the expanding tarsus and cytoplasmic in the pretarsus (pt). (F, F') ~4 h After Pupal Formation (APF) and (G, G') ~8 h APF; in everting discs, Myc-Lines was nuclear in the tarsus and in the inter-joint region of the tibia (ti), and cytoplasmic in the pretarsus, and in the tarsal/tibial joint. (H) ~72 h; Flag-Bowl was broadly nuclear. (I) ~84 h; Flag-Bowl was downregulated in the nascent central fold. (J) ~120 h; Flag-Bowl was stabilized in nuclei in three rings that correspond to the presumptive pretarsa/tarsal, tarsal/tibial and tibial/femural joints. (K, K') ~4 h APF; Bowl was stable in the proximal half of the t5/pretarsal and the tibial/t1 joints. (L, M) Bowl. (N, O) *odd-lacZ*. (L) ~84h; Bowl was broadly expressed at early stages of leg disc development. Yellow bars and lines highlight the broad uninterrupted pattern of Bowl accumulation in proximal cells. Red bars and arrowheads point to the nascent tarsus where Bowl was downregulated. (M) ~120 h; Yellow bar highlight the accumulation of Bowl is six rings that correspond to the Notch-activated region of true leg segments. Red bars point to the tarsal region. (N) ~84 h and (O) ~120h; an *odd-lacZ* reporter was expressed in a similar pattern to Bowl. (N) The *odd-lacZ* reporter was broadly expressed at early stages. (O) At later stages, the *odd-lacZ* reporter was maintained in 6 rings that correspond to the Notch-activated region of each true leg segment as indicated with yellow bars and asterisks. Scale bar = 30 µm in C, H, L, N, O, 40 µm in F, K, K', M, 50 µm in D, E, H, I, 80 µm in G, G'.

medial and distal domains along the leg PD axis (Abu-Shaar and Mann, 1998; Azpiazu and Morata, 2002; Campbell and Tomlinson, 1998; Cohen et al., 1989, 1993; Gorfinkiel et al., 1997; Mardon et al., 1994; Wu and Cohen, 1999), the tarsal PD genes act locally to fine-pattern the tarsal region.

Distinct combinations of leg gap genes and tarsal PD genes initiate the expression of *Dl* and *Ser* across each prospective leg segment either directly or indirectly (Rauskolb, 2001). At the end of larval development, *Dl* and *Ser* are expressed at the distal end of each prospective leg segment and signal asymmetrically to distal cells to control the expression of downstream target genes that mediate leg segment growth and joint morphogenesis (Bishop et al., 1999; de Celis et al., 1998; Mishra et al., 2001; Rauskolb and Irvine, 1999). Downstream of Notch are the *Drosophila transcriptional activator protein 2* (*dAP-2*), *nubbin* (*nub*) and the *odd-skipped* family genes *drumstick* (*drm*), *odd-skipped* (*odd*), *bowl*, and *sister of odd and bowl* (*sob*). *dAP-2* mediates growth of all the leg segments and the formation of all the joints (Kerber et al., 2001; Monge et al., 2001). *nub* encodes a POU domain protein whose role in leg development is yet to be elucidated. *nub* hypomorphs develop shortened and gnarled legs indicating that *nub* contributes to the growth and patterning of leg segments (Cifuentes and Garcia-Bellido, 1997). The *odd-skipped* family genes, *drm*, *odd*, *sob* and *bowl*, share a highly conserved zinc finger domain (Coulter et al., 1990; Green et al., 2002; Hart et al., 1996; Wang and Coulter, 1996) and are induced by Notch signaling along the borders of true leg segments (de Celis Ibeas and Bray, 2003; Hao et al., 2003). The nuclear protein Lines acts reciprocally to Drm and Bowl in patterning embryonic and larval structures (Bras-Pereira and Casares, 2006; Green et al., 2002; Hatini et al., 2005; Johansen et al., 2003; Nusinow et al., 2008). Lines destabilizes Bowl by binding to the Bowl protein, while Drm stabilizes Bowl by binding and restricting Lines to the cytoplasm (Green et al., 2002; Hatini et al., 2005). The analysis of *lines* and *bowl* function in several tissues have led to a model whereby the two genes act as a binary switch to subdivide a field of cells into adjacent domains (Fig. 1B). *bowl* had been reported to specify distal and proximal tarsal fates and to inhibit medial tarsal fates. *bowl* had also been reported to mediate the morphogenesis of true joints but its role in this process had not been investigated (de Celis Ibeas and Bray, 2003). Given the relationship between *lines* and *bowl* in other tissues, we sought to understand how *lines* might complement the activity of *bowl* in patterning the tarsus, how *lines* and *bowl* might contribute to the patterning, growth and morphogenesis of true leg segments, and whether *lines* and *bowl* might play a general role in ventral appendage development.

We find that Lines is broadly cytoplasmic and thus inactive while Bowl is broadly nuclear and thus active throughout the leg disc at early stages. The progressive segmental subdivision of the leg disc correlates with the segmental accumulation of Lines in nuclei and a corresponding segmental destabilization of the Bowl protein. Across the emerging tarsus, Lines accumulates in nuclei

where it modulates the opposing expression landscapes of *dac* and the tarsal PD genes. In this manner, Lines inhibits proximal tarsal fate and promotes medial and distal tarsal fates. Across emerging proximal segments, *lines* promotes *Dl* expression by destabilizing Bowl using a relief-of-repression mechanism. *Dl*, then, acts to maintain Bowl expression in adjacent distal cells. In turn, *bowl*, together with one or more redundant factors, acts cell-autonomously to repress *Dl* expression in the Notch-activated region. This regulatory feedback mechanism generates a stable Notch signaling interface at segment borders. Our results lead us to extend and revise previous models of tarsal and segmental patterning. Finally, we uncovered analogous roles for *lines* in developing antennae, which are serially homologous to legs, revealing fundamental roles for *lines* in ventral appendage formation.

Material and methods

Genetics and fly strains

UAS-N$^{[rk111]\Delta34}$ (C. Rauskolb), *UAS-Dl*, *UAS-Myc-Lines (8)* (weak insertion), *UAS-Lines (9.2)* (strong insertion), *UAS-LinesRNAi* (16801, VDRC), *UAS-BowlRNAi* (3775, VDRC), *UAS-DrmEst (2.1)* and *UAS-Flag-Bowl (29)* were expressed in clones using a combination of the FLP/FRT and the UAS/GAL4 techniques (Pignoni and Zipursky, 1997), or in restricted domains using the GAL4/UAS technique (Brand and Perrimon, 1993) with *ptc-GAL4; UAS-GFP*, *Dll-GAL4*^{md23}, *bab-GAL4*$^{Agal4-2}$, *bab-GAL4*$^{Agal4-5}$, *rn-GAL4*$^{GAL4-5}$, *rn-GAL4*$^{GAL4\text{-}DeltaS}$ and *dac-GAL4*^{P7d23} and *klu-GAL4*. The *lines*^{2f}, *lines*^{G2}, *drm*^{3}, *odd*^{rk111}, and *bowl*^{1} alleles were used to generate mutant clones using the FLP/FRT (Xu and Rubin, 1993), or the MARCM techniques (Lee and Luo, 2001). The following FRT-carrying chromosomes were used: *w; 42DFRT Ubi-GFP/CyO* (B. Edgar), *y w hs-FLP122*, *Tub-GAL4*, *UAS-GFP; 42DFRT Tub-Gal80 hs-CD2*, *y*$^{+}$*/CyO* (G. Struhl); *y w hs-FLP122*, *Tub-GAL4*, *UAS-GFP; Tub-Gal80 40AFRT/CyO; y w hs-FLP122; 42DFRT lines*^{G2}*/CyO;* and *bowl*^{1} *FRT40A/CyO*. The *42DFRT Tub-Gal80 CD2*, *y*$^{+}$ chromosome was used to identify *lines* mutant clones in adult flies. The following reporters were used: *Bar-lacZ*$^{B\text{-}H2P058}$, *ap-lacZ*^{UG62}, *odd-lacZ*^{rk111}, *Dl-lacZ* and *bib-lacZ*^{4163}.

Immunofluorescence and imaging

Eggs were collected in a drop of live yeast on grape agar plates and aged at 25 °C for 60 h, 72 h, 84 h, 96 h or 120 h to examine the dynamic distribution of Myc-Lines, Flag-Bowl, Bowl and an *odd-lacZ* reporter. Discs were fixed and stained according to standard protocols using rabbit anti-Bowl (S. Bray), rabbit anti-dAP-2 (D1; P. Mitchell), mouse anti-Nub (2DAb7; S. Cohen), mouse anti-Dll (S. Cohen), guinea pig anti-Hth (R. Mann), rabbit anti-β-galactosidase (Cappel), rabbit anti-BarH1 (T. Kojima), rat anti-Bab2 (A. Laski), mouse anti-Dac, mouse anti-Dl and rat anti-Ci (DSHB), rat anti-C15 and rat anti-Al (G. Campbell) and guinea pig anti-dLim1 (J. Botas). Secondary antibodies conjugated to the Cy2, Cy3 and Cy5 fluorophores (Jackson ImmunoResearch) were used at 1:150. Nuclei were stained with Hoechst 33258 (Molecular Probes). Stained discs were scanned using a Zeiss LSM510 confocal microscope in multi-tracking mode. Adult legs were imaged using a Zeiss Axioscope 2+ and reconstructed using composite ZP. Images were assembled and adjusted using Adobe Photoshop CS3.

Results

Lines accumulates in nuclei across the emerging tarsal primordium and the inter-joints of proximal leg segments

The activities of the Lines and Bowl proteins are regulated by post-translational mechanisms. Lines accumulates in nuclei where it is active and in the cytoplasm where it is repressed. Reciprocally, Bowl is unstable where Lines is active and stable where Lines is repressed (Hatini et al., 2000, 2005; Nusinow et al., 2008). To understand how *lines* contributes to leg development, we first examined the dynamics of Lines distribution relative to Bowl in developing leg imaginal discs. To do the analysis, we expressed a weak Myc-Lines transgene (UAS-Myc-Lines 8) and separately a weak Flag-Bowl transgene (UAS-Flag-Bowl 29) in a broad central domain using the *Dll-GAL4* driver and examined the dynamic subcellular distribution of the tagged proteins. The ectopic expression of Myc-Lines and Flag-Bowl did not alter the morphology of adult legs suggesting that the tagged proteins were regulated by post-translational mechanisms that regulate the abundance and distribution of the endogenous proteins. At the early third larval stages, Lines was cytoplasmic, and thus inactive, in a broad central domain (Figs. 1C–C'). By the mid-third instar, Myc-Lines was detected in nuclei in the central fold in the emerging tarsal primordium (Figs. 1D–D'). At later stages, Myc-Lines remained nuclear in the tarsus (Figs. 1E–F'). In addition, Myc-Lines accumulated in nuclei in emerging inter-joints of proximal leg segments, and remained enriched in the cytoplasm in the pretarsus and presumptive true joints (e.g. ti/ta joint; Figs. 1F–G'). Expression of Myc-Lines with *dac-GAL4* across the intermediate region of the leg disc revealed nuclear accumulation of Myc-Lines across the inter-joints of the tibia, femur and trochanter and cytoplasmic accumulation across true joints (Fig. S1C and data not shown). *klumpfuss* (*klu*)*-GAL4* is expressed across the tarsus, the inter-joint of each leg segment and a narrow stripe immediately distal to the segment border. Expression of Myc-Lines with *klu-GAL4* revealed a nuclear accumulation of Lines across the tarsus and across the inter-joint of each leg segments and a cytoplasmic accumulation in a narrow stripe immediately distal to the segment border (Fig. S1B). In contrast, *lines* transcripts were detected broadly and at a uniform level consistent with the notion that *lines* is primarily regulated by post-translational mechanisms (Fig. S1A). The accumulation of Myc-Lines in nuclei coincided with the formation of the tarsus and each proximal leg segment suggesting an important role for *lines* in leg segmentation.

Lines destabilizes Bowl across the growing tarsus and the inter-joints of proximal leg segments

The Flag-Bowl protein was enriched in nuclei in a roughly complementary pattern to Myc-Lines. By the early third instar, Flag-Bowl was detected in nuclei in a broad central domain where Lines was cytoplasmic (Fig. 1H). At the mid-third instar, Bowl was down-regulated in a circumferential domain in the nascent central fold (Fig. 1I) where Lines accumulated in nuclei. At later stages, Flag-Bowl was stable in the proximal half of true joints where Lines was cytoplasmic (Figs. 1J–K'). However, Flag-Bowl was unstable across the tarsal field and across the growing inter-joints of proximal segments where Lines was nuclear. Flag-Bowl was also unstable in the pretarsus and in the distal half of true joints where Lines was cytoplasmic suggesting that additional mechanisms modulate the activities of Lines and Bowl in these regions.

Analysis of Bowl accumulation with an antibody that recognizes the endogenous protein revealed a broad accumulation of Bowl at early stages (data not shown). This was followed by the loss of Bowl in a circular domain across the nascent tarsus and subsequently across each proximal leg segment (Figs. 1L, M, respectively). The spatiotemporal expression pattern of an *odd-lacZ* reporter was similar to that of Bowl (Figs. 1N, O). Collectively, these findings are consistent with a model whereby the formation of leg segments is dependent on the segmental repression of *odd-skipped* related genes and the coincidental segmental activation of Lines.

The roughly complementary distributions of Lines and Bowl suggested that *lines* destabilizes Bowl across the tarsal primordium and the inter-joints of true segments. Consistent with this idea, Bowl was stabilized cell-autonomously in *lines* mutant clones that were induced in inter-joint territories (Figs. 5D–D") (Hatini et al., 2005), and was destabilized in the *Ptc* domain or in FLP-out clones expressing a strong *UAS-lines* transgene (see Figs. S2A–A' in supplementary material and data not shown). During embryogenesis, Drm stabilizes Bowl by inhibiting Lines (Hatini et al., 2005). Similarly, Bowl was stabilized ectopically across presumptive inter-joints in cells expressing *drm* with *Ptc-GAL4* (Figs. 5G–G'). However, Bowl was stable in *drm* mutant clones (Figs. S2B–B") suggesting that *drm* acts with other redundant factor/s to stabilize Bowl across presumptive joints. The dynamic pattern of Lines and Bowl distribution was largely complementary suggesting reciprocal roles for *lines* and *bowl* in tarsal and segmental patterning.

lines promotes the growth and patterning of the tarsus and the proximal leg segments

To test the contribution of *lines* to the formation of the tarsus, we expressed a *UAS-linesRNAi* transgene using the tarsal-specific *bab-GAL4* and *rn-GAL4* drivers, and the more broadly expressed *Dll-GAL4* and *dac-GAL4* drivers. We also removed *lines* function using the FLP/FRT and the MARCM techniques in genetically marked clones (Golic, 1991; Xu and Rubin, 1993; Lee and Luo, 2001). Subsequently, we analyzed the resulting phenotypes in adult legs. We focused the analysis on male prothoracic legs in which the proximal tarsal segment 1 (t1) is decorated at its base with a row of darkly pigmented sex comb (sc) bristles (Fig. 2A). Expression of *linesRNAi* with *bab-Gal4*, *rn-GAL4* and *Dll-GAL4* resulted in a phenotypic series in which progressively more distal tarsal segments were fused with t1 and

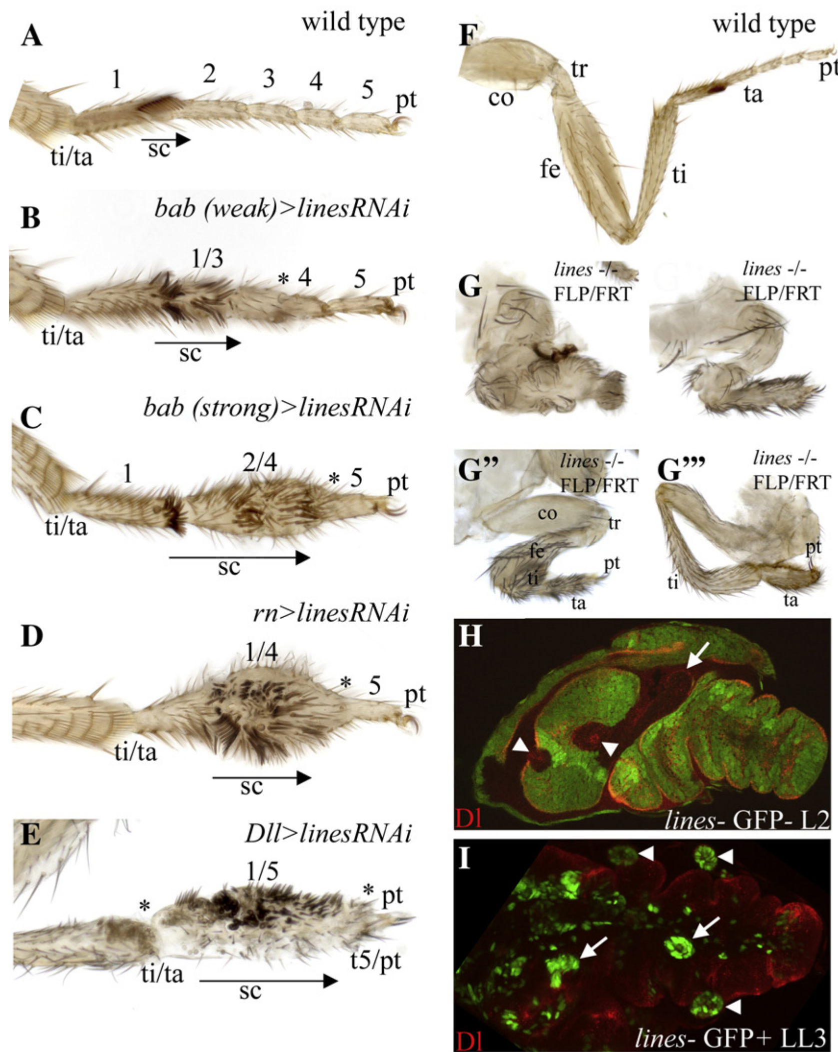

Fig. 2. The loss of *lines* function disrupts the formation of the tarsus and the growth and morphogenesis of true leg segments. (A–E) Tarsi of adult prothoracic legs; ti/ta— tibial/tarsal joint, 1–5— tarsal subsegments t1–t5, pt— pretarsus, sc— sex comb. Arrows indicate the scope of the region that differentiates sex comb bristles and assumes t1 identity, *—fused joint. (A) Wild type; the tarsus is subdivided into five jointed subsegments. The pretarsus forms the claw. (B) *bab* (weak)>*linesRNAi*, (C) *bab* (strong)>*linesRNAi* and (D) *rn*>*linesRNAi* (strong). Broad expression of *linesRNAi* across the tarsal primordium with various *GAL4* drivers led to a phenotypic series in which progressively more distal segments fused with t1 and differentiated sex comb bristles indicating assumed t1 identity. (E) *Dll*>*linesRNAi*; most severe phenotype. t1–t5 were fused and sex comb bristles differentiated along the fused tarsus. In addition, the ti/ta and t5/pt joints were malformed and associated with internal necrotic vesicles. Bristle orientation in the tibia was randomized. (F) Wild type. (G–G") Legs with *lines* MARCM clones induced at the second instar caused deep invaginations and a severe reduction in the growth of the tarsus and the proximal leg segments. (G"') Clones induced at the early third instar led to reduced growth and segmentation of the tarsus and the bending of true leg segments. Images in panels F and G–G"' are shown at equal magnification. (H) *lines* FLP/FRT clones induced at second instar (48–72 h after egg laying) were recovered in the tarsus and in proximal segments. However, the clones disrupted the shape and size of leg segments in a cell-autonomous manner and blocked the formation of the segment border as reflected by the loss of Dl expression. Arrow points to tibial clone that caused a reduction in tibial size and loss of Dl expression. Arrowhead point to two smaller clones that appeared to sort out from surrounding cells. (I) *lines* MARCM clone induced at the late third instar (96–120 AEL); the clones formed round vesicles with smooth borders that segregated from the DP and accumulated both below (arrows) and above (arrows) the surface epithelium.

differentiated sex comb bristles indicating that distal tarsal cells assumed t1 identity (Figs. 2B–E compare to wild type in Fig. 2A). Expression of *linesRNAi* with *Dll-GAL4* led to the most severe tarsal phenotype — the fusion of all the tarsal segments and the differentiation of sex comb bristles along the fused tarsus (Fig. 2E).

The expression of *linesRNAi* with *dac-GAL4* along the intermediate region of the leg disc led to a severe decrease in size of true segments and loss of true joints (Figs. S1E–E", compare to wild type in S1D–D"; asterisks mark fused joints). These phenotypes suggested additional roles for *lines* in controlling the growth of proximal segments and the formation of true joints. In addition, cuticle decorated with bristles was replaced with naked cuticle and numerous vesicles were detected under the basal surface of the epithelium within the leg shaft (Figs. S1E–E").

To explore the role of *lines* in leg development in further detail we examined the effect of *lines* mutant clones on leg development. *lines* mutant clones induced at the second instar led to the shortening and fusion of all the proximal segments and the formation of deep invaginations in the cuticle (Figs. 2G–G"). Clones that were induced at the early third instar led to the shortening of the tarsus and the fusion of tarsal segments (Fig. 2G'"). However, *lines* mutant clones marked by the loss of a *yellow*$^{+}$ transgene in a *yellow*$^{-}$ background were not recovered suggesting that the *lines* mutant cells failed to contribute to inter-joint tissue or to differentiate inter-segmental *yellow*$^{-}$ bristles consistent with the phenotype of the *dac*>*linesRNAi* legs described above. We occasionally observed out-pocketing of epithelial tissue decorated with smooth cuticle at inter-joint territories suggesting that at least a subset of the *lines* mutant clones survived to adult stages, sorted out from the epithelium and failed to differentiate bristles (Fig. S1F, arrow indicates a positively marked *lines* MARCM clone). The over-expression of *lines* in cell clones led to the accumulation of melanotic tissue near true leg joints (Fig. S1G). Altogether, these loss- and gain-of-function phenotypes implicated *lines* in the formation, growth and patterning of each leg segment.

Analysis of clone recovery and sorting behavior in developing imaginal discs revealed that the *lines* mutant clones induced at the second instar were recovered at a similar rate to control clones. However, these clones appeared to cause a reduction in the size of proximal leg segments (Fig. 1H, arrow points to a tibial clones that caused a cell-autonomous reduction in tibial size). Similarly, clones that were induced at the early and third larval stages survived to late larval and early pupal stages and their morphology was dependent on their position across both tarsal and proximal leg segments. Clones that were induced in the native Bowl domain assumed a normal elongated shape comparable to the morphology of control clones (Fig. 5D', arrow). In contrast, clones that were induced adjacent to the endogenous Bowl domain assumed an abnormal rounded shape with smooth borders and thus sorted apart from surrounding wild type cells (Fig. 5D', arrowhead). Similarly, *lines* MARCM clones induced at the third instar and analyzed at early pupal stages either extruded inwards from the basal surface of the epithelium into the disc lumen (Fig. 2I, arrows), or outwards from the apical surface (Fig. 2I, arrowheads). Below we examined the *lines* loss- and gain-of-function phenotypes using molecular markers to delineate the pathways in which *lines* acts.

lines specifies distal and medial tarsal fates and inhibits the specification of proximal tarsal fates

de Celis Ibeas and Bray (2003) had previously reported that *bowl* promotes proximal and distal tarsal fates and inhibits medial tarsal fates. If *lines* acts reciprocally to *bowl*, *lines* should promote medial tarsal fates and repress proximal and distal tarsal fates. To test this prediction, we examined the expression of the leg gap gene *dac* and the tarsal PD genes, *bab2* and *BarH1* (referred to as *bab* and *Bar*) in *Dll*>*linesRNAi* leg discs. These genes are expressed in broad overlapping PD domains across the tarsal region, and their expression pattern roughly corresponds to the regions affected by their absence. *dac* mediates the formation of the femur, tibia and the three proximal tarsomeres (Mardon et al., 1994). *bab 1* and *2* mediate the formation of tarsal segments 2 to 5 (Couderc et al., 2002; Godt et al., 1993; St Pierre et al., 2002) while *BarH1* and *2* (*Bar*) mediate the formation of tarsal segments 3–5 (Kojima et al., 2000). We found distal expansion of Dac, loss of Bab, and distal retraction of Bar expression in these discs (Figs. 3D–F" compare to wild type in A–C"). The coordinated changes in expression of Dac and the tarsal PD genes corresponded nicely to the patterning defects seen in adult *Dll*>*linesRNAi* legs (Fig. 2E). To confirm these results, we examined the expression of these genes in *lines* mutant clones induced at the second instar using the FLP/FRT technique. We found that Bab was lost in all the *lines* mutant clones (Hatini et al., 2005), and Bar was downregulated in the proximal region of the Bar domain (Figs. 3G–G" and H–H", respectively). The tarsal PD gene *ap* is expressed in tarsal segment 4 and controls the proper development of this segment (Kojima et al., 2000). Similar to the regulation of *bab* and *bar* by *lines*, the expression of an *ap-lacZ* reporter was repressed in *drm*-expressing clones in which the activity of *lines* was inhibited (Fig. S3A). Conversely, Dac was ectopically expressed in tarsal clones that were induced between the native Dac domain and the central fold (Figs. 3I–I").

To determine if *lines* was sufficient to influence tarsal patterning, we examined the expression of these proteins in genetically marked clones expressing a strong *UAS-lines* transgene. We found ectopic expression of Bab, Bar and *ap-lacZ* in clones that were induced near their respective expression domains (Figs. 3J–J", K–K" and Figs. S3B–C, respectively). Conversely, we found repression of Dac in clones spanning the distal region of the Dac domain (Figs. 3L–L").

Finally, we found that *bowl* mediated the *lines* clonal phenotypes because Bab was maintained in *lines bowlRNAi* clones (Fig. S2C). Thus, contrary to our expectation, we found that *lines* promotes the specification of both distal (marked by *ap* and Bar) and medial (marked by Bab) tarsal fates and inhibits the specification of proximal tarsal fate (marked by Dac). We therefore propose an extension, and a partial revision, to the model proposed by de Celis Ibeas and Bray (2003) whereby *bowl* promotes the specification of proximal tarsal fates only, and *lines* antagonizes *bowl* across the emerging tarsal primordium, proximal to the central fold, to allow expression of tarsal PD genes by a gradient of EGF receptor signaling that emanate from the distal tip of the leg disc (see Fig. 3M for a schematic depiction of the model).

To determine if *lines* contributes to the primary subdivision of the leg PD axis, we also examined the expression of Dll and Hth in *lines* mutant clones. We found no change in expression of Dll in the clones indicating that *lines* acts either downstream or in parallel to *Dll* to pattern the tarsus (Fig. S4A). Similarly, we found no change in expression of Hth indicating that *lines* is not involved in PD patterning of the disc periphery (Fig. S4B). Finally, we also examined the expression of the pretarsal proteins dLim1, C15 and Aristaless (Al) (Campbell, 2005; Campbell et al., 1993; Kojima et al., 2005; Lilly et al., 1999; Schneitz et al., 1993; Tsuji et al., 2000), whose expression is dependent on high levels of EGFR signaling (Campbell, 2002; Galindo et al., 2002). We detected relatively normal levels of these proteins in the clones indicating that *lines* does not affect the expression of EGFR ligands or the activation of EGF receptor signaling (see Fig. S4C, and data not shown).

lines maintains Dl expression across proximal leg segments to maintain a stable Notch signaling interface at leg segment borders

In the absence of *lines* function true leg segments were reduced in size and joints were lost as shown above (Figs. 2G–G" and S1E–E"). We hypothesize that, similar to its segment polarity role in embryos, *lines* could participate in patterning each true leg segment. A key step in

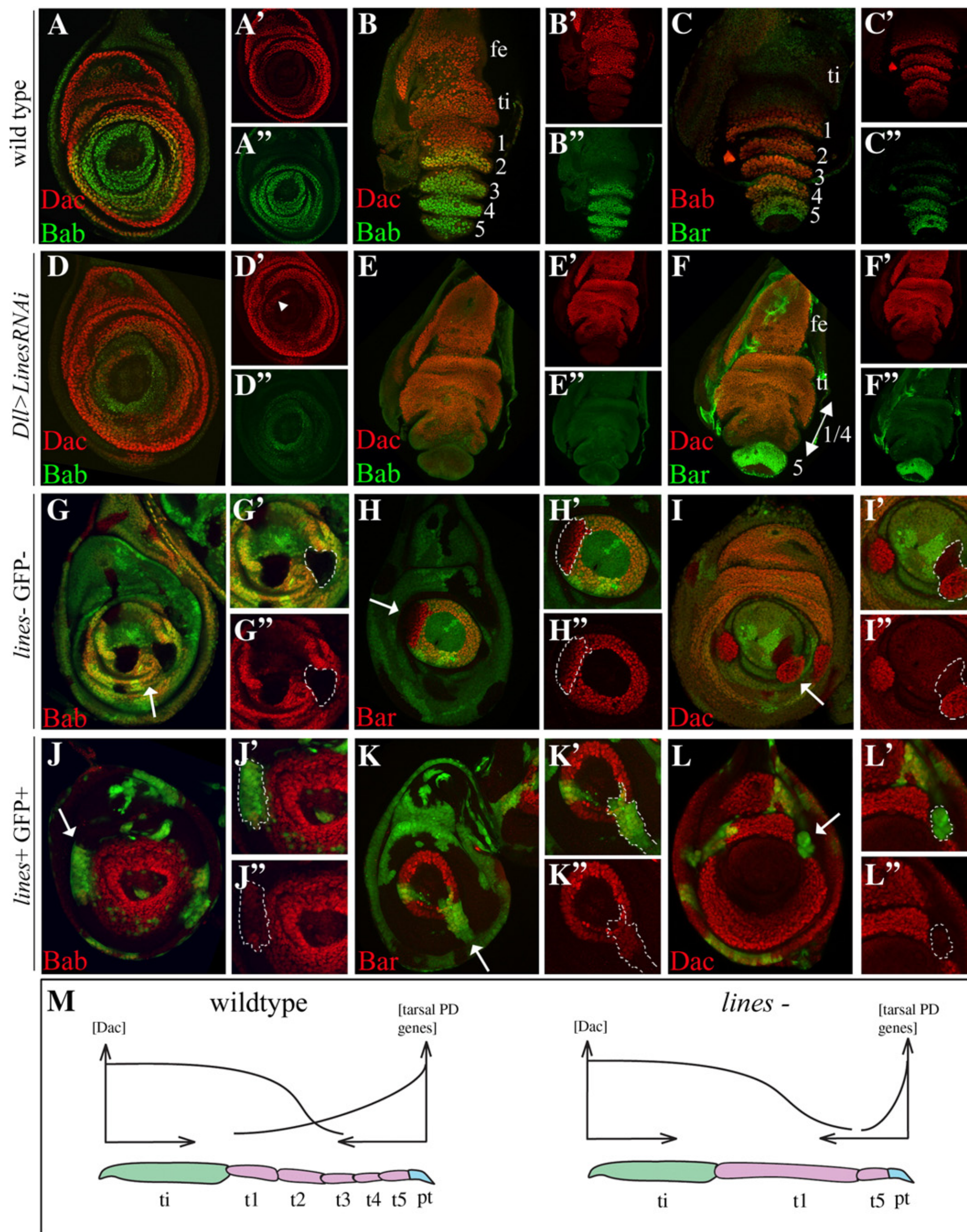

Fig. 3. *lines* specifies distal and medial tarsal fates and inhibits the specification of proximal tarsal fate. (A–C") Wild type. (D–F") *Dll*>*linesRNAi*. (G–I") *lines* FLP/FRT clones. (J–L") *lines* expressing FLP-out clones. Boundaries of selected clones were outlined by dashes for ease of identification. Arrows in G–L point to magnified areas shown in insets. (A–B") Dac and Bab and (C–C") Bab and Bar are expressed in broad nested domains. (A–B") Dac levels are high in the tibia, t1 and t2 and lower in t3. (A–C") Bab expression is high in t4 and t3 and progressively lower in t2 and t1; (C–C") Bar is high in t5 and progressively lower in t4 and t3; (D–F") *Dll*>*linesRNAi*, (D–D") third instar and (E–F") everting discs; (D–F") Dac expression expanded distally. Note ectopic Dac in distal cells in panel D (arrowhead); (D–E") Bab was lost; (F–F") Bar was retracted distally. Similarly, in *lines* mutant clones (G–G") Bab was lost, and (H–H") Bar was downregulated in the proximal region of the Bar domain. (I–I") Dac was ectopically expressed between the Dac domain and the central fold in *lines* expressing clones, (J–J") Bab and (K–K") Bar were ectopically expressed adjacent and near their respective domains, and (L–L") Dac was repressed in the proximal region of the Dac domain. (M) A model depicting the contribution of *dac*, the tarsal PD genes and *lines* to tarsal patterning and segmentation. *lines* represses *dac* expression and promotes expression of tarsal PD genes proximal to the central fold, which marks the boundary between the pretarsus and tarsal segment 5 and the remaining proximal tarsomeres. See text for further detail.

segmental patterning is the initiation of *Dl* and *Ser* expression across each leg segment and the establishment of a stable border between *Dl*/*Ser*-expressing cells and adjacent distal cells. Dl and Ser signal across this border to induce expression of target genes, which further mediate segmental growth and joint formation. In addition, these targets participate in a negative feedback regulation to repress *Dl* and *Ser* expression cell-autonomously to stabilize the segment border (Ciechanska et al., 2007; Shirai et al., 2007). Notch pathway activation promotes Bowl accumulation in the Notch-activated region (Campbell, 2005; de Celis Ibeas and Bray, 2003). In turn, Bowl may act to repress *Dl* and *Ser* cell-autonomously to stabilize the segment border. Reciprocally, *lines* may antagonize *bowl* to maintain *Dl* and *Ser* expression in proximally adjacent cells by relief-of-repression (see Fig. 4J for a model). If this model is correct, the accumulation of Bowl in *lines* mutant clones that form within the *Dl*/*Ser* domain could lead to the repression of Notch ligands in the clones. Dl and Ser produced by surrounding wild type cells may then be permitted to induce expression of Notch targets in the clones.

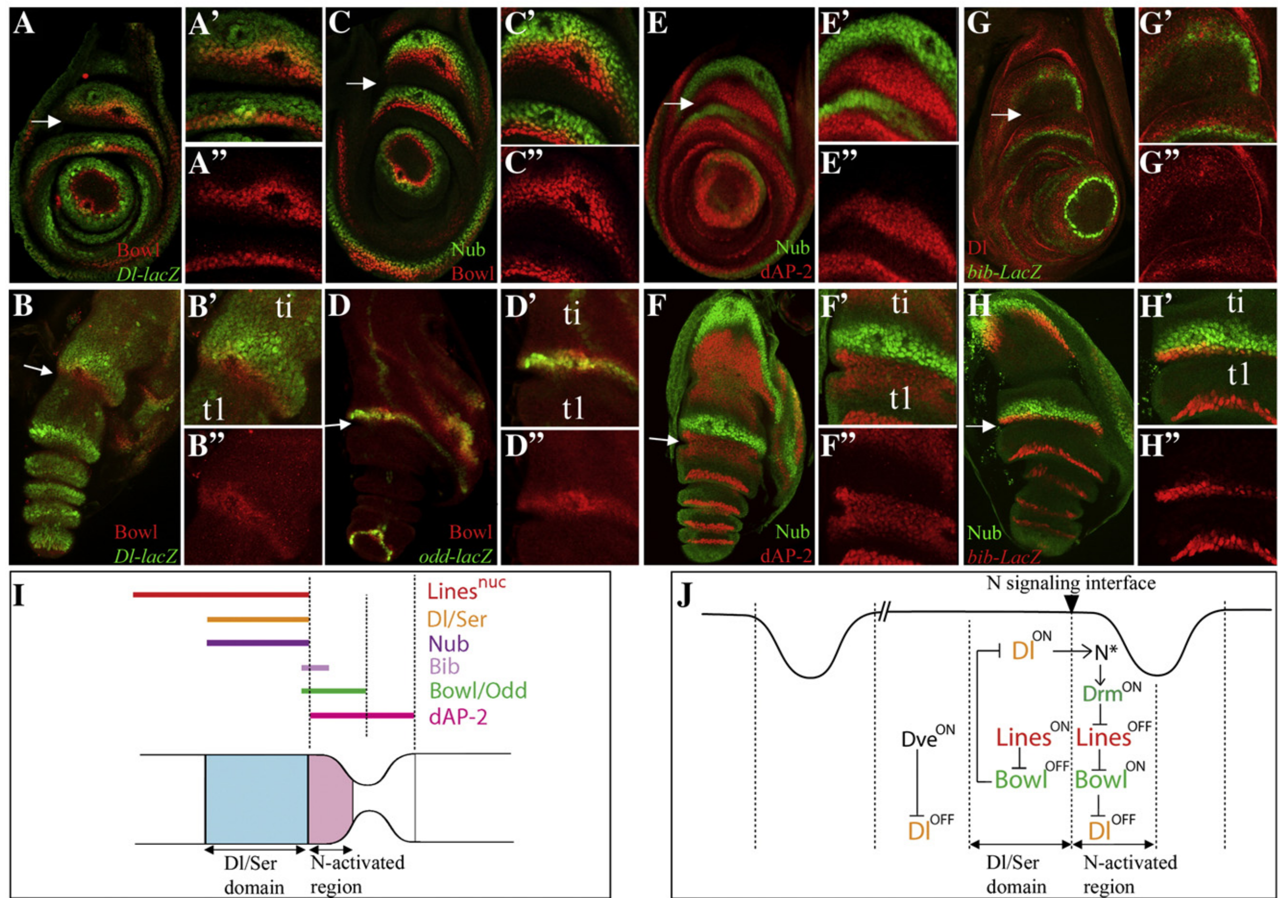

Fig. 4. Expression of *Dl* and Notch targets defines multiple domains across true leg segments. (A, C, E, G) Late third instar. (B, D, F, H) Everting pupal legs. Leg discs were double labeled to map domains of gene expression. Dl, *Dl-lacZ*, dAP-2 and *bib-lacZ* were detected in all the leg segments, while Bowl, *odd-lacZ* and Nub were detected in true leg segments only. (A–B") Bowl was detected in the proximal half of true joints distal to the *Dl-lacZ* domain in a partially overlapping domain. (C–C") Bowl was detected distal to Nub in a partially overlapping domain. (D–D") *odd-lacZ* was co-expressed with Bowl in the proximal half of joint constrictions. (E–F") dAP-2 was detected distally adjacent to the Nub domain in a broad region that spanned the joint constriction. (G–H") *bib-lacZ* was detected along the segment border distal to Dl (G–G") and Nub (H, H') in a partially overlapping domain. (I) A cartoon depicting the expression of *Dl* and Notch targets relative to the tibial/tarsal joint in everting legs. (J) A model depicting the interactions that regulate *Dl* expression across true leg segments. *dve* represses *Dl* expression proximal to *Dl*-expressing cells and Bowl together with one or more redundant factor represses *Dl* expression in the Notch-activated region. Lines antagonizes Bowl to maintain *Dl* expression in proximal cells. Together, *lines* and *bowl* act as a binary switch to maintain a stable Notch signaling interface at the distal end of each leg segment.

To address this hypothesis, we first examined the relative expression of the Notch ligand Dl and the Notch targets Bowl, Odd, Nub, dAP-2, and Bib in wild type leg discs at late stages of leg development. While Dl, dAP-2 and Bib are expressed across both tarsal and true leg segments, Bowl, Odd and Nub are only expressed across true leg segments (Fig. 4). At late third instar and in everting leg discs, a *Dl-lacZ* reporter was detected at the distal end of leg segments and Bowl was detected in a distal and a slightly overlapping domain in the proximal half of presumptive joints (Figs. 4A, B). Similarly, an *odd-lacZ* reporter was co-expressed with Bowl in this region (Fig. 4D) (de Celis Ibeas and Bray, 2003). The expression pattern of Nub and dAP-2 differed significantly from that of Bowl. Similar to *Dl-lacZ*, Nub was expressed proximal to Bowl in a partially overlapping domain (Fig. 4C). By the late third larval stage, Nub and dAP-2 were detected in adjacent non-overlapping domains (Fig. 4E). In everting legs, Nub was detected in a narrow domain just proximal to the presumptive joint (e.g. ti/ta joint), and dAP-2 was broadly expressed across the joint constriction (Fig. 4F). Bib, a member of the aquaporin family of channel proteins, is required for the reception of the Notch signal and is upregulated in Notch-activated cells (Doherty et al., 1997; Rao et al., 1990). A *bib-lacZ* reporter was upregulated at the distal edge of Dl and Nub-expressing cells along the segment border (Figs. 4G–H"). Thus, at late larval and early pupal stages, Bowl (and Odd), dAP-2, Bib, and Nub are each expressed in a different domain relative to the segment border (see Fig. 4I for a schematic depiction of the segment border). The co-expression of Nub and Dl is surprising given that Nub is a Notch target (Rauskolb and Irvine, 1999) and the *Dl*/*Ser*-expressing cells are believed to be refractory to Notch signaling. The expression of *bib-lacZ* along the segment border appears to mark cells that respond to Notch signaling. Bowl and dAP-2 are both detected across several cell diameters distal to the Notch signaling interface suggesting that their expression at a distance from this interface is maintained by auto-regulatory mechanisms.

To determine if *lines* contributes to the formation of segment borders (see Fig. 4J for a model), we examined the expression of the Dl ligand and a *Dl-lacZ* reporter in *lines* mutant clones. The expression of Dl and *Dl-lacZ* was either lost or reduced in *lines* mutant clones in both tarsal and non-tarsal segments (Figs. 5A–A" and B–B", respectively), which reflects the dual role that *lines* plays in tarsal and segmental patterning. The downregulation of Dl and *Dl-lacZ* expression in tarsal clones (indicated in asterisks in Figs. 5A, B) results from changes in expression of Dac and the tarsal PD genes, which mediate tarsal segmentation. The loss of *Dl* expression in proximal clones could reflect a second role for *lines* in maintaining *Dl* expression and thus a stable Notch signaling interface across true leg segments.

To further test this hypothesis, we examined the expression of the Notch targets, Bowl, Nub, dAP-2 and Bib in *lines* mutant clones. We detected downregulation of Nub in clones that spanned the

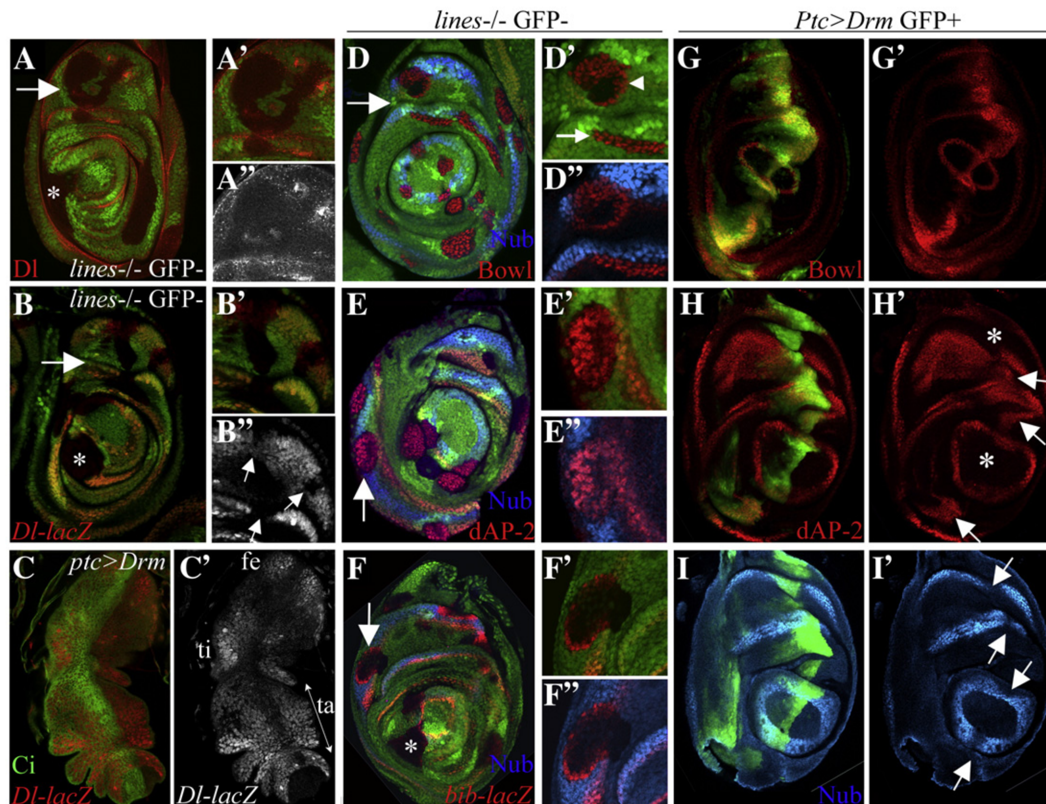

Fig. 5. *lines* maintains *Dl* expression across inter-joint territories and inhibits formation of ectopic segment borders. (A–B", D–F") *lines* FLP/FRT clones marked by the absence of GFP. (C, C', G–I") *ptc*>*Drm*; the *ptc* domains is marked by high Ci levels in C, and by GFP in G–I. Arrows in A, B and D–F point to magnified areas shown in insets. Asterisk in A, B and F indicates tarsal clones. (A) Dl and (B) *Dl-lacZ* were downregulated in *lines* mutant clones generated across the tarsus and across proximal leg segments. (C) *Dl-lacZ* was downregulated in *drm*-expressing cells. (D) Bowl was ectopically expressed and Nub was repressed in all the *lines* mutant clones. (E) Nub was repressed and dAP-2 was ectopically expressed in most of the *lines* mutant clones that were induced in the Nub domain. (F) The ring pattern of *bib-lacZ* expression was disrupted in *lines* mutant tarsal clones (asterisk). In addition, *bib-lacZ* accumulated ectopically in *lines* mutant clones that were induced in the Nub domain along clone borders. (G, G') Bowl, and (H, H') dAP-2 were ectopically expressed and (I, I') Nub was repressed (arrows) in *Ptc*>*drm*-expressing cells. dAP-2 was not induced in the pretarsus and was only weakly induced distal to the tibia in *Ptc*>*drm*-expressing cell (asterisks).

endogenous Nub domain (Figs. 5D–F"). Reciprocally, we detected ectopic cell-autonomous induction of Bowl in all the clones (Figs. 5D–D"). Similarly, we detected ectopic dAP-2 expression in most clones that were induced in the Nub domain (Figs. 5E–E"). The *bib* gene is induced in a narrow stripe along the segment border in the Notch-activated region as shown above (Figs. 4G–H"). We detected perturbation of *bib* expression in tarsal clones (asterisks in Fig. 5F points to a tarsal clone) reflecting the role *lines* plays in tarsal segmentation as discussed above. In addition, we detected ectopic *bib* expression along the borders of *lines* mutant clones that were induced in the Nub domain of true segments (Figs. 5F'–F"). We propose that the loss of Dl expression in the *lines* mutant clones permitted Dl produced by surrounding wild type cells to induce expression of Notch targets in the clones. These results further support the idea that *lines* controls the formation of segment borders.

To determine if *bowl* mediates the *lines* clonal phenotype, we ectopically expressed *drm* with *Ptc-GAL4* to block Lines and stabilize Bowl (Figs. 5G–G'). We found that *drm* was sufficient to downregulate the expression of *Dl-lacZ* and Nub (Figs. 5C–C' and I–I', respectively) and to promote the expression of dAP-2 (Figs. 5H–H') across inter-joints. To test this idea directly, we examined Dl expression in *lines bowlRNAi* clones. We found no change in Dl expression in the clones indicating that *bowl* represses *Dl* expression and *lines* inhibits *bowl* to promote *Dl* expression by a relief-of-repression mechanism (Fig. S2D).

If *bowl* represses *Dl* expression in the Notch-activated region, the loss of *bowl* function or the ectopic expression of *lines* should disrupt the formation of the segment border. To test this idea, we examined the expression of dAP-2 and Nub in *bowl* MARCM clones and in *lines* FLP-out clones. We found no change in expression of these markers in most clones. However, in a small number of clones we detected downregulation of dAP-2 and upregulation of Nub along the segment border (Fig. S2E–E", F–F", respectively, and data not shown). These changes in gene expression suggest that *bowl* contributes to the formation of a stable segment border although it is not absolutely essential. The low incidence of these phenotypes suggests that *bowl* acts redundantly with one or more factors, possibly *odd* and/or *sob*, to stabilize the segment border. The *drmP2* deficiency removes *drm*, *sob* and *odd* and approximately 30 other genes. We therefore attempted to generate *drmP2* mutant clones to determine if the three genes act redundantly to stabilize the segment border. However, we failed to recover *drmP2* clones in either a wild type or a Minute background (data not shown). In addition, we generated MARCM *bowl oddRNAi* clones but observed no changes in expression of dAP-2 or Nub in these clones (data not shown). Thus, additional work will be required to identify the combination of factors that act redundantly to repress *Dl* expression in the Notch-activated region.

lines organizes PD and segmental patterning in developing antennal imaginal discs

The *Drosophila* antennae are serially homologous to legs and have been considered to evolve from an ancestral leg-like appendage by the activity of homeotic and field-specific selector genes (Schneuwly et al., 1987; Shubin et al., 1997; Casares and Mann, 1998, 2001). To determine if *lines* is generally required to mediate ventral appendage

development, we examined the dynamic sequence of Lines distribution relative to Bowl in developing antennae, and the contribution of *lines* to antennal PD and segmental patterning.

The antennal appendage is composed of the proximal AI–AIII segments, the basal cylinder (bc) and the distal arista (ar) (Fig. 6A). At the early third instar, a Myc-Lines transgene expressed with *Dll-GAL4* was enriched in the cytoplasm in a broad central domain (Fig. 6E). By the mid-third instar, Myc-Lines appeared in nuclei at the distal tip of the antenna and in the cytoplasm in surrounding proximal cells (red bar in Fig. 6F). At late larval and early pupal stages, Myc-Lines was nuclear in a broader distal region corresponding to the presumptive AIII, the basal cylinder and the arista, and cytoplasmic in the adjacent proximal region (Figs. 6G and H, respectively). While Lines accumulated in nuclei at the distal tip of the antenna (Fig. 6H), it accumulated in the cytoplasm at the distal tip of the leg (Fig. 1D) revealing a variation in the regulation of Lines between legs and antennae or the lack of equivalent tissue in antennae. We also expressed UAS-Flag-Bowl with *Dll-GAL4* to examine the pattern of Bowl stabilization relative to Myc-Lines. By the early third instar, Bowl was nuclear at the distal region of the disc where Lines was cytoplasmic (Fig. 6I). At later stages, Flag-Bowl was lost from the distal tip, but was nuclear in surrounding proximal cells where Lines was cytoplasmic (Figs. 6J–L). Similarly, the Bowl protein was broadly nuclear at early stages (data not shown). At later stages, Bowl was lost from a circular domain at the distal tip of the antenna (Fig. S5A) and subsequently in three rings corresponding to the inter-joints of antennal segments AI–AIII (Fig. S5B).

To investigate the contribution of *lines* to antennal PD patterning, we expressed a *linesRNAi* transgene with *Dll-GAL4* and found that AIII, the basal cylinder and the arista were replaced with a poorly differentiated tubular structure (Fig. 6B). The expression of *linesRNAi* with *bab-GAL4* led to poor differentiation and expansion of the basal cylinder and the aristal stalk (Fig. 6C). The expression of *linesRNAi* with *rn-GAL4* distorted the morphology of AIII and led to a poor differentiation of the basal cylinder (Fig. 6D). The observed morphological malformations were restricted to the regions where each driver was expressed. *lines* mutant clones induced at the second instar led to similar malformations in adult antennae (not shown). Thus, *lines* patterns a broad distal domain in which it localizes to nuclei. To further explore the role of *lines* in antennal PD patterning, we examined the expression of Bab, *rn-LacZ* and Dac in *lines* mutant clones. We detected loss of Bab and *rn-LacZ* (Figs. 6M–M" and N–N",

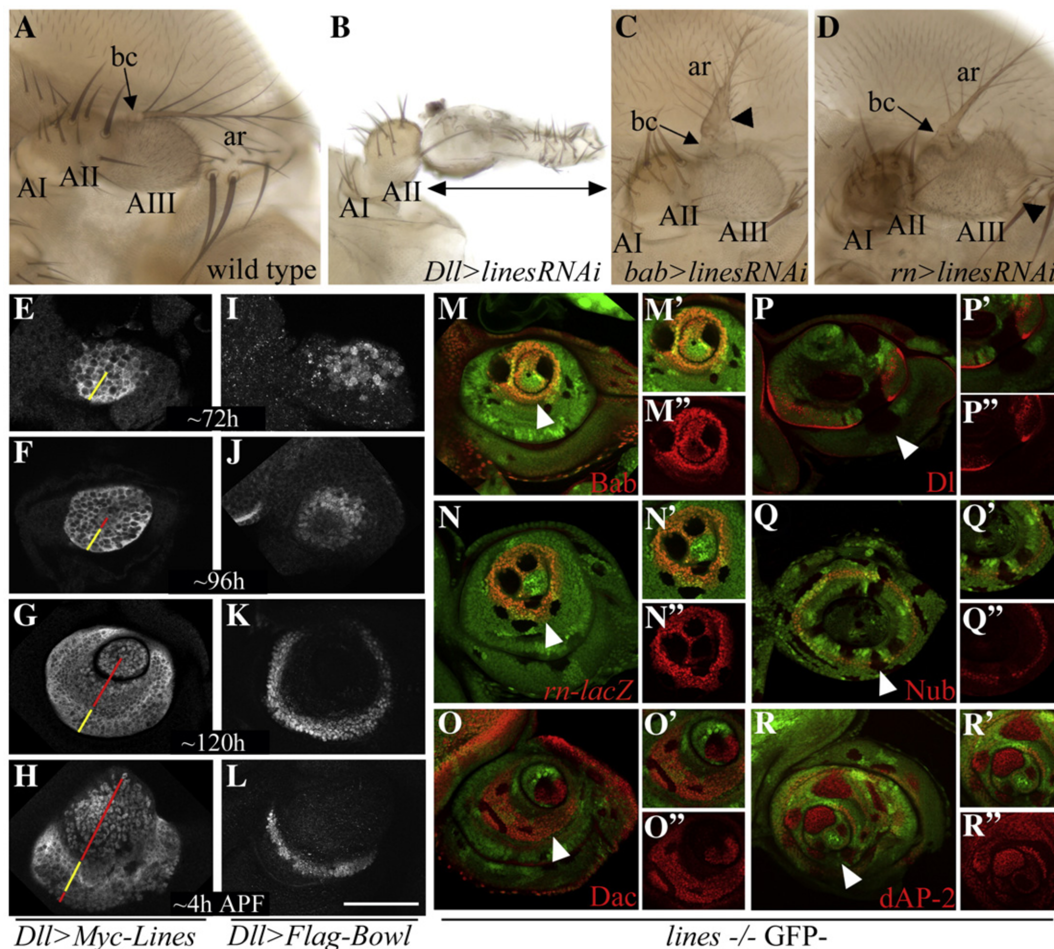

Fig. 6. *lines* contributes to antennal PD and segmental patterning. (A–D) Adult antennae. (E–H) *Dll>Myc-Lines;* yellow and red bars mark regions where Lines was enriched in the cytoplasm and nucleus, respectively. (I–L) *Dll>Flag-Bowl.* (M–R) *lines* mutant FLP/FRT clones marked by the absence of GFP and stained for PD (M–O") and segmental markers (P–R"). (A) Adult antennae consist of the AI–AIII segments, the basal cylinder (bc) and the arista (ar). (B) *Dll>linesRNAi*; AIII and the arista were replaced with an elongated tubular structure (double arrow) that was poorly differentiated. (C) *bab>linesRNAi*; the basal cylinder (arrow) and the aristal stalk (arrowhead) were expanded, malformed and poorly differentiated. (D) *rn>linesRNAi*; AIII and the basal cylinder were malformed. (E) ~72 h; Myc-Lines was cytoplasmic in a broad central domain; (F) ~96 h; a new domain where Lines accumulates in nuclei emerged at the distal tip. (G, H) ~120 h and ~4 h APF, respectively; this domain expanded to encompass the presumptive AIII, basal cylinder and the arista. Myc-Lines also appeared in nuclei in emerging antennal segments. (I–L) Bowl accumulated in a reciprocal pattern to Lines. (I) ~72 h; Bowl was nuclear in a broad distal domain where Lines was cytoplasmic. (J) ~96 h; Bowl was unstable in the distal tip of the antenna where Lines was nuclear. (K–L) ~120 h and ~4 h APF; Bowl was stabilized in presumptive AII/AIII joints. The endogenous Bowl protein is detected in three concentric rings that correspond to the three antennal joints (not shown). (M) Bab and (N) *rn-lacZ* were lost in *lines* mutant clones. (O) Dac was ectopically expressed distal to the Dac domain and was lost in the endogenous Dac domain. (P) Dl, and (Q) Nub were downregulated and (R) dAP-2 was ectopically expressed in most of the *lines* mutant clone. Scale bar = 20 μm in E, I, 50 μm in F, J, H, L, 75 μm in G, K.

respectively) and ectopic Dac expression in *lines* mutant clones that were induced distal to the endogenous Dac domain (Figs. 6O–O"). *rn* was also lost in *lines* mutant clones induced in the leg imaginal disc (data not shown). We also detected repression of Dac in the endogenous Dac domain (Figs. 6O–O") revealing a dual role for *lines* in repressing Dac expression distally and maintaining its expression medially. In the leg, *lines* does not maintain Dac expression medially, revealing a variation between the function of *lines* in legs and antennae. Similarly, we detected a near complete loss of Bab expression and upregulation of Dac expression near the distal tip in *Dll*>*linesRNAi* antennal discs (data not shown). To analyze the role of *lines* in patterning proximal antennal segments, we examined the expression of Dl, Nub and dAP-2 in *lines* mutant clones. We detected downregulation of Dl and Nub and ectopic dAP-2 expression in the clones across both distal and proximal antennal segments (Figs. 6P–P", Q–Q", R–R", respectively). We infer that *lines* maintains *Dl* expression to generate a stable Notch signaling interface across each antennal segment. Our data suggests that *lines* plays analogous roles in both leg and antennal development. However, we also find significant variations in the regulation and function of *lines* between legs and antennae that may have contributed to the evolution of distinct ventral appendage morphologies.

Discussion

We assign two crucial roles for *lines* in patterning the tarsal PD axis and in patterning true leg segments. Lines accumulates in nuclei in the emerging tarsal primordium where it antagonizes *bowl* to specify medial and distal tarsal fates and inhibit proximal tarsal fates. In addition, Lines accumulates in nuclei across inter-joints of true leg segments where it antagonizes *bowl* to promote *Dl* expression. We provide evidence from misexpression analysis and genetic epistasis to suggest that *bowl*, together with one or more redundant factor possibly *odd* and *sob*, acts reciprocally to *lines* to repress *Dl* expression in the Notch-activated region in order to maintain a stable Notch signaling interface between *Dl*-expressing cells and adjacent distal cells. Finally, we assign analogous roles for *lines* in patterning the antennal imaginal disc. We propose central roles for *lines* in mediating leg and antennal segmentation and consider a possible evolutionarily conserved role for *odd-skipped* genes in arthropod and vertebrate limb development.

lines modulates the expression levels of the leg gap gene dac and the tarsal PD genes bab, ap and bar to mediate tarsal patterning and segmentation

At the mid-third larval stage, Lines accumulates in nuclei in a circumferential domain in the emerging tarsal primordium (Figs. 1D, E). In *lines* deficient legs, tarsal segments 1–5 were fused and distal cells assumed proximal t1 identity (Fig. 2E) indicating that *lines* specifies medial and distal tarsal fates. The leg gap gene *dac* and the tarsal PD genes mediate tarsal patterning and segmentation. Dac is distributed in a modest proximal to distal gradient with high levels in the tibia, t1 and t2 and lower levels in t3 (Figs. 3A, B) (Mardon et al., 1994). The tarsal PD genes are distributed in a modest distal to proximal gradient (Figs. 3A–C) (Couderc et al., 2002; Godt et al., 1993; Kojima et al., 2000; St Pierre et al., 2002). The graded expression of the tarsal PD genes is established by a gradient of EGFR signaling generated by the secretion of EGF receptor ligands from the distal tip of the leg (Campbell, 2002; Galindo et al., 2002). Reciprocal cross-regulatory interactions between these genes further refine their expression domains. We find that *lines* is both necessary and sufficient to repress *dac* expression and to promote the expression of the tarsal PD genes *bab*, *ap* and *bar* across the tarsal primordium. By expressing a *linesRNAi* transgene with various *GAL4* drivers, we obtained a phenotypic series that reveals a higher sensitivity of t2 and progressively lower sensitivities of t3 and t4 towards transformation into t1 (Figs. 2B–E) indicating that the region most sensitive to fate transformation is where the opposing expression landscapes of *dac* and the tarsal PD genes intersect. We, thus, infer that *lines* modulates these expression landscapes to establish cell type diversity across the tarsal field and to mediate tarsal segmentation (see Fig. 3M for a model). *bowl* has been proposed to repress *ap* expression to subdivide the distal limb field into smaller domains (Campbell, 2005). However, the observation that Bowl and Ap are expressed several cell diameters apart from one another makes it unlikely for Bowl to directly repress *ap* expression and inconsistent with this model. We favor an alternative model whereby Bowl acts to generally repress the expression of the tarsal PD genes at early stages, whereas Lines destabilizes Bowl at later stages to permit expression of the tarsal PD genes by relief-of-repression. This model is consistent with the dynamic expression pattern of Lines and Bowl in the emerging tarsal field and with the general role that *lines* plays in regulating the expression of the tarsal PD genes as reported in this study.

lines deficient legs develop a simple tarsus that resembles the unsegmented tarsus of primitive arthropods (Snodgrass, 1935). It is therefore conceivable that *bowl* mediates the formation of the ancestral unsegmented form of the tarsus, a function reflected in the stabilization of Bowl in a broad domain at early stages. The activation of Lines within the nascent tarsus may reflect a more recent evolutionary change that enabled the formation of additional tarsal segments found in higher arthropods. Phylogenetic comparisons of the regulation and function of *lines* and *odd-skipped* genes in tarsal patterning will be required to evaluate this model.

lines and odd-skipped related genes may act as a binary switch to maintain a stable Notch signaling interface at segment borders

In *lines* deficient legs, the proximal leg segments were severely reduced in size and joints were lost (Figs. 2G–G" and Fig. S1E–E"). A key step in the formation of leg segments is the initiation of *Dl* and *Ser* expression across each leg segment and the generation of a Notch signaling interface between the *Dl*/*Ser* domain and the adjacent distal domain (Bishop et al., 1999; de Celis et al., 1998; Mishra et al., 2001; Rauskolb and Irvine, 1999). The formation of a stable segment border depends on multiple levels of control. The gene *defective proventriculus* (*dve*) represses *Dl* expression in the proximal part of each leg segment (Ciechanska et al., 2007), while the glycosyl transferase *fringe* (*fng*) can modulate binding between Notch and its ligands in the *Dl*/*Ser* domain or proximal to it (Fleming et al., 1997; Panin et al., 1997). In addition, *Dl* and *Ser* can autonomously inhibit Notch activation within the *Dl*/*Ser* domain (de Celis and Bray, 1997; Doherty et al., 1996; Klein et al., 1997; Micchelli et al., 1997). Finally, Notch targets, such as *dAP-2*, can repress *Dl* and *Ser* expression in the Notch-activated region to stabilize the segment border using negative feedback regulation (Ciechanska et al., 2007; Shirai et al., 2007).

Our studies provide evidence that *lines* and *odd-skipped related* genes *drm* and *bowl* participate in a gene regulatory network to generate the segment border. Following the activation of *Dl* proximal to the presumptive joint, Dl signals to adjacent distal cells to activate *drm* (and one or more redundant factors, possibly *odd* and/or *sob*) (Hao et al., 2003). Drm, in turn, acts cell-autonomously to inhibit Lines thereby allowing Bowl to accumulate in the distal region of each true leg segment. Bowl, then, functions to repress *Dl* expression in this region. Reciprocally, Lines antagonizes Bowl to maintain *Dl* expression in adjacent proximal cells. Together, *lines* and *bowl* (and one or more redundant factors) act to generate and maintain a stable Notch signaling interface between *Dl*-expressing cells and adjacent distal cells (see Fig. 4J for a model). Factors induced along this interface are believed to mediate the growth of leg segments and the morphogenesis of leg joints.

In early leg discs, Bowl and *odd* are broadly expressed. The formation of leg segments correlates with the segmental down-regulation of Bowl and *odd* expression and the coincidental accumulation of Lines in nuclei. It is plausible that the leg gap gene *hth*, *dac* and *Dll* act combinatorially to repress the expression of the *odd-skipped* genes and to initiate the segmental expression of *Dl* and *Ser* using a relief-of-repression mechanism. We provide evidence that *Dl*, *Ser* and *odd-skipped* genes, in turn, regulate each other's expression to stabilize and maintain the segment border. This model predicts that cis-regulatory modules in the *odd-skipped* related genes *drm*, *sob* and/or *odd* are configured to respond to the repressive activities of certain combinations of the leg gap proteins, while cis-regulatory modules in *Dl* and Ser are configured to respond to the repressive activities of Odd-skipped proteins.

While the morphogenesis of tarsal joints depends on Jun Kinase (JNK)-*reaper*-dependent apoptosis (Manjón et al., 2007), the mechanisms that generate true joints are not known. *odd-skipped* family genes have been proposed to initiate the cytoskeletal rearrangements that mediate leg joint morphogenesis at the border between leg segments by controlling the expression of putative cytoskeletal regulators (Hao et al., 2003). Our findings suggest instead that *odd-skipped related* genes influence epithelial morphology rather indirectly by stabilizing the Notch signaling interface at segment borders (Fig. 5). Our findings, however, do not exclude the possibility that *odd-skipped* related genes might act in a parallel pathway to control joint morphogenesis. It is conceivable that the special mechanical properties of the interface between Bowl-expressing cells and the adjacent distal cells buckle the epithelium along this border to initiate joint morphogenesis. While Bowl accumulates in the proximal half of true joints (Figs. 1K', 4B–B", D–D"), dAP-2 is expressed uniformly across prospective joints (Figs. 4F–F"). dAP-2 may therefore act to modulate cell adhesion and cytoskeletal dynamics to remodel the topology of prospective joints. The phenotype of *dAP-2* mutant legs is consistent with such a role (Ciechanska et al., 2007; Kerber et al., 2001; Monge et al., 2001). Understanding how segmental patterning is coordinated with epithelial morphogenesis will necessitate the identification of the genes that directly regulate epithelial morphogenesis.

During antennal development, *bowl* inhibits the formation of ectopic antennae by repressing *wg* expression in dorsal cells (Brás-Pereira and Casares, 2008). Loss of *bowl* function, however, does not cause defects in antennal PD patterning or in antennal segmentation. Given that *lines* affects both antennal PD patterning and antennal segmentation (Fig. 6) and given the relationship between *lines* and *bowl* in other tissues, we would predict that *bowl* acts together with one or more redundant factors reciprocally to *lines* to pattern the antennal PD axis and mediate antennal segmentation.

Evolutionary perspective

The antennae, feeding appendages, legs and genitalia are very different in structure and function but have been considered to diverge from an ancestral ventral appendage on the basis of comparative anatomical and molecular studies (Shubin et al., 1997). The analogous contribution of *lines* to leg and antennal development suggests an evolutionary conserved role for *lines* in ventral appendage formation. The variations in the regulation and function of *lines* and *odd-skipped* genes between legs and antennae may have contributed to the morphological diversity of these appendages. It is further intriguing to consider the possibility that Odd-skipped related (Osr) genes had been deployed in the common ancestor of arthropods and vertebrates, and had evolved to perform analogous roles in limb development in both phyla. Consistent with this idea, at early stages, vertebrate Osr1 and Osr2 are expressed in broad PD domains across the nascent limb bud, while at later stages their expression shifts to sites of synovial joint formation (Stricker et al., 2006). The striking similarity in the dynamic expression of *odd-skipped* family genes in flies and vertebrate limb development may reflect a remarkable case of convergent evolution or a common origin in developmental pathways that are now deployed in both phyla. Comparative phylogenetic studies will be required to explore this question.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2009.03.014.

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