Bowl is required downstream of Notch for elaboration of distal limb patterning

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Summary

In the *Drosophila* leg, activation of Notch leads to the establishment of the joints that subdivide the appendage into segments. We find that mutations in *bowl* result in similar phenotypes to Notch, causing fusion and truncations of tarsal segments (tarsomeres) and, like its close relative Odd-skipped, Bowl is produced in response to Notch signalling at a subset of segment boundaries. However, despite the fact that *bowl* mutant clones result in fusion of tarsomeres, Bowl protein is only found at the t1/tibial and t5/pretarsal boundaries, not at tarsomere joints. One hypothesis to reconcile these data is that *bowl* has a role at an earlier stage in tarsal development. We therefore investigated the effects of *bowl* mutations on the expression of leg 'gap' genes that confer regional identity on the developing leg. Several of these genes have altered expression in *bowl* mutant cells. For example, *bric-a-brac2* is normally expressed in the central part of the tarsus domain but expands into distal and proximal regions in *bowl* clones. Conversely, ectopic *bowl* leads to a reduction in *bric-a-brac2*, with a concomitant expansion of proximal (t1) and distal (t5) tarsomere fates. The *bowl* gene is therefore required for the elaboration of pattern in the tarsus and its effects suggest a progressive model for the determination of P/D identities. This mechanism might be important in the diversification of arthropod limbs, because it explains how segmented tarsomeres could have arisen from an ancestral limb with an unsegmented tarsus.

Key words: Limb development, Tarsus, *Drosophila*, *Notch*, *odd-skipped*, Zinc-finger protein

Introduction

Animal limbs develop as outgrowths from the main body axis that acquire proximal/distal (P/D) patterning to form a series of specialized skeletal structures. These structures are articulated and so one key consequence of P/D patterning is the establishment of joints between each skeletal element. In the *Drosophila* leg, the P/D axis is established through the combined activities of Wingless (Wg) and Decapentaplegic (Dpp), which intersect in the centre of the limb primordium. Wg and Dpp together induce the expression of Distal-less (Dll), a homeodomain protein required for the development of all distal leg structures (Cohen et al., 1989; Diaz-Benjumea et al., 1994), and Dachshund (Dac), a nuclear protein required for intermediate leg segments (femur and tibia) (Mardon et al., 1994; Lecuit and Cohen, 1997). By the beginning of the third larval instar, the leg primordium is therefore subdivided into at least three regions. Subsequent patterning involves interactions between the factors expressed in these early territories. For example, several genes are required for the development of the tarsus, including *rotund* and *bric-a-brac* (Kerridge and Thomas-Cavallin, 1988; Agnel et al., 1989; Godt et al., 1993; Chu et al., 2002; Couderc et al., 2002; Galindo et al., 2002; St Pierre et al., 2002). Expression of these genes is promoted by Dll and restricted by the combined activities of Dac proximally and a gradient of epidermal-growth-factor-receptor signalling distally (Campbell and Tomlinson, 1998; Campbell, 2002; Galindo et al., 2002). By the stage that a series of P/D regions have been established, further patterning appears to be independent of the initial inducers Wg and Dpp (Lecuit and Cohen, 1997; Galindo et al., 2002). However, it is not clear how these P/D regions are elaborated (for example, to give diversity to the distal tarsal structures).

A final stage in translating the P/D patterning into the definitive segmented structure of the insect adult leg is the formation of the inter-segmental joints. The leg consists of six true segments or podites (coxa, trochanter, femur, tibia, tarsus and pretarsus), which are independently moveable by muscles. In *Drosophila*, the tarsus is further subdivided into five tarsomeres (t1-t5), which have distinct characteristics but lack independent musculature (Snodgrass, 1935). Development of both 'true' joints and inter-tarsomere joints requires Notch activity, shown by the loss of joints and fused segments in *Notch* mutant cells, and by the ectopic joints that are formed when extra sites of Notch activity are engineered (de Celis et al., 1998; Bishop et al., 1999; Rauskolb and Irvine, 1999). Consistent with its pivotal role in specifying joint development, Notch activity is detected at all segment/subsegment boundaries at the end of larval development, using transcription of the *Enhancer of split* target genes as a measure (de Celis et al., 1998). However, expression of Notch ligands is first observed at a subset of locations at a much earlier stage shortly after the initial 'regional' domains of gene expression are established (Rauskolb, 2001). There are two explanations for this. One is that the specification of joints occurs sequentially, with some joints being determined early and

others (e.g. tarsomere joints) much later. Alternatively, Notch activity might have both earlier roles in P/D regionalization and patterning and later roles that build on these earlier events to establish the segmental boundaries and joints at the correct locations.

To investigate further the mechanisms involved in P/D limb development, we have looked for genes whose expression is dependent on Notch activity that could allow us to establish whether it has roles in the initial P/D patterning as well as in the subsequent establishment of joints. The zinc-finger protein encoded by the gene *bowl* is detected at a subset of sites of Notch activity and its expression is dependent on Notch. The *bowl* gene is closely related to the segmentation gene *odd-skipped*, and is required for development of the embryonic hindgut (Wang and Coulter, 1996; Iwaki et al., 2001). Our analysis of *bowl* and *odd-skipped* function in the developing leg indicates that these genes are involved in the elaboration of pattern in the tarsus, leading us to propose that Notch is important for patterning as well as for joint formation. The effects of Bowl on tarsal development suggest that P/D tarsal identities are determined progressively and might also explain how different numbers of tarsomeres could have arisen from an ancestral limb that is thought to have contained an unsegmented tarsus (Snodgrass, 1935).

Materials and methods

Genetics

Except where otherwise stated, fly stocks used are described in FlyBase. For analysis of Bowl, we used both *bowl*^{2}, a strong loss-of-function allele (H284Y) (Wang and Coulter, 1996) and *bowl*^{1}, a null allele (S232@) (Wang and Coulter, 1996). We also analysed three P elements inserted within 100 bp of the transcription start site, including *bowl*^{k08617}. None correspond to the *bowl* alleles because they fully complement *bowl*^{2} and *Df(2R)ed*^{1}. The *lacZ/Gal4* pattern of expression in imaginal discs also differs between each P element, so we have not used these further. The *lacZ* insertion in *odd* is *P{lacZ}odd*^{rK111} and *odd*^{5} is a strong hypomorph. *E(spl)mβ-CD2* and *E(spl)mβ1.5-lacZ* are reporter genes that mimic *E(spl)mβ* transcription (de Celis et al., 1998).

The *bowl* or *odd* alleles were recombined onto *w*^{1118}*; P{mW*$^{+mw}$*=pM}36F P{ry*$^{+t7.2}$*=neoFRT}40A* and were crossed to the following marked strains for inducing clones:
f^{36a} hsFLP; ck {f^{+}} P{ry$^{+t7.2}$=neoFRT}40A/CyO
f^{36a} hsFLP; P{ry^{+} y^{+}}25F P{ry$^{+t7.2}$=neoFRT}40A/CyO
f^{36a} hsFLP; P{ubi-GFP} P{ry$^{+t7.2}$=neoFRT}40A

Clones were induced by a 1-hour heat shock at 38°C at 48-72 hours of development. Large mutant clones were also induced by X-irradiation at a dose rate of 1.28 Rads per second for 900 seconds using the Minute stock *f*^{36a}*; M(2)z P{f+}30B/CyO*.

Notch mutant clones were generated using directed FLPase expression by crossing the stocks *N*^{81k} *P{ry*$^{+t7.2}$*=neoFRT}101* / FM7 females with *y w GFP*^{XI} *P{ry*$^{+t7.2}$*=neoFRT}101/Y; ptc-Gal4; UAS-FLPase/ SM6a-TM6B* males (gift of T. Klein and A. Martinez-Arias).

UAS-bowl was generated using the full-length cDNA clone LD15614 obtained from the Berkeley *Drosophila* Genome Project. The *bowl* cDNA was excised using *Not*I and *Xho*I, and ligated into pUAST digested with the same enzymes. Transgenic flies were obtained by injection into *y w*, following standard P-element transformation procedures. Independent lines were analysed and graded according to the strength of phenotypes elicited with GAL4 drivers as follows: *UAS-bowl*$^{1.1}$ (strong) > *UAS-bowl*$^{6.1}$ (moderate) > *UAS-bowl*$^{9.1}$ (weak)

Immunofluorescence

Leg discs were dissected from wandering third-instar larvae and from pupae (up to 18 hours after pupation). Indirect immunofluorescence was carried out as previously described (de Celis et al., 1998). Anti-Bowl antibodies were generated in rabbits by Sigma Genosys. Peptides used for immunization were [Cys]-PPIAPPPAPPRRT-GFSIEDIMRR and [Cys]-DLPRVHDLPREEDDD-FDPEDEEQ. Anti-Bowl serum was used at a final dilution of 1/1000. Other primary antibodies were rabbit anti- GFP (Molecular Probes), 1:1000; rabbit anti-BarH1 (Higashijima et al., 1992), 1:1000; rabbit anti-β-galactosidase (Cappel), 1:500; rabbit anti-Serrate (Thomas et al., 1991), 1:20; rat anti-Bab2 (Couderc et al., 2002), 1:2000; and mouse anti-Dac, 1:5, and anti-Dl, 1:5 [developed by Kooh et al. (Kooh et al., 1993) and Mardon et al. (Mardon et al., 1994), respectively, and obtained from the Developmental Studies Hybridoma Bank, University of Iowa, Department of Biological Sciences].

Results

Bowl and Notch produce similar segmentation and growth defects in the leg

In the developing leg, *Notch* mutant cells result in fused leg segments, owing to the absence of joints, and in severely reduced growth (Fig. 1A-C) (Shellenbarger and Mohler, 1978; de Celis et al., 1998; Bishop et al., 1999; Rauskolb and Irvine, 1999). Accumulation of pigmented tissue also occurs at joints in proximal regions (data not shown). Mutations in *bowl* result in similar phenotypes; the mutant cells are associated with fusions and truncations of tarsal segments as well as with melanotic patches at the proximal joints (Fig. 1D-F; Fig. 2). Because the gene is essential at earlier stages in development (*bowl* mutant embryos do not hatch; Wang and Coulter, 1996), it is difficult to determine the consequence of completely eliminating *bowl* in the leg. However, when the mutant clones cover most of the distal part of the leg, the limb is severely truncated, with little or no segmentation/joint tissue evident (Fig. 1E and data not shown). Conversely, clones that are restricted to the central part of the tarsus often fail to result in a detectable phenotype (Fig. 2).

The *bowl* gene encodes a zinc-finger transcription factor and is closely related to *odd-skipped*, a gene that has already been implicated in leg segmentation (Wang and Coulter, 1996; Rauskolb and Irvine, 1999). We therefore examined phenotypes produced by *odd* mutant cells and found similar defects to *bowl* – segmental fusions and truncations in the tarsal region, and melanotic patches in proximal joints (Fig. 1G,H). The two genes therefore have similar but essential roles in leg segmentation, although their functions elsewhere appear to be distinct (Wang and Coulter, 1996).

Given the profound effect on tarsal segmentation and similarities with *Notch* phenotypes, we expected that *bowl* would be expressed at the sites where Notch is active in the tarsus. We therefore compared the expression of *bowl* with *E(spl)mβ*, a known target of Notch signalling in the leg, using an *E(spl)mβ-lacZ* transgene (Cooper et al., 2000) and an antibody that recognizes Bowl. Although Bowl and β-galactosidase are clearly co-expressed at some positions, including the t5/pretarsus boundary and the tibia/t1 boundary, Bowl was not detected at sites of Notch activity within the tarsus (Fig. 1I-I″). Indeed, the distribution of Bowl and Odd appears to be identical and neither is detected at tarsomere boundaries (Fig. 1J-K″) (Rauskolb and Irvine, 1999). Both are

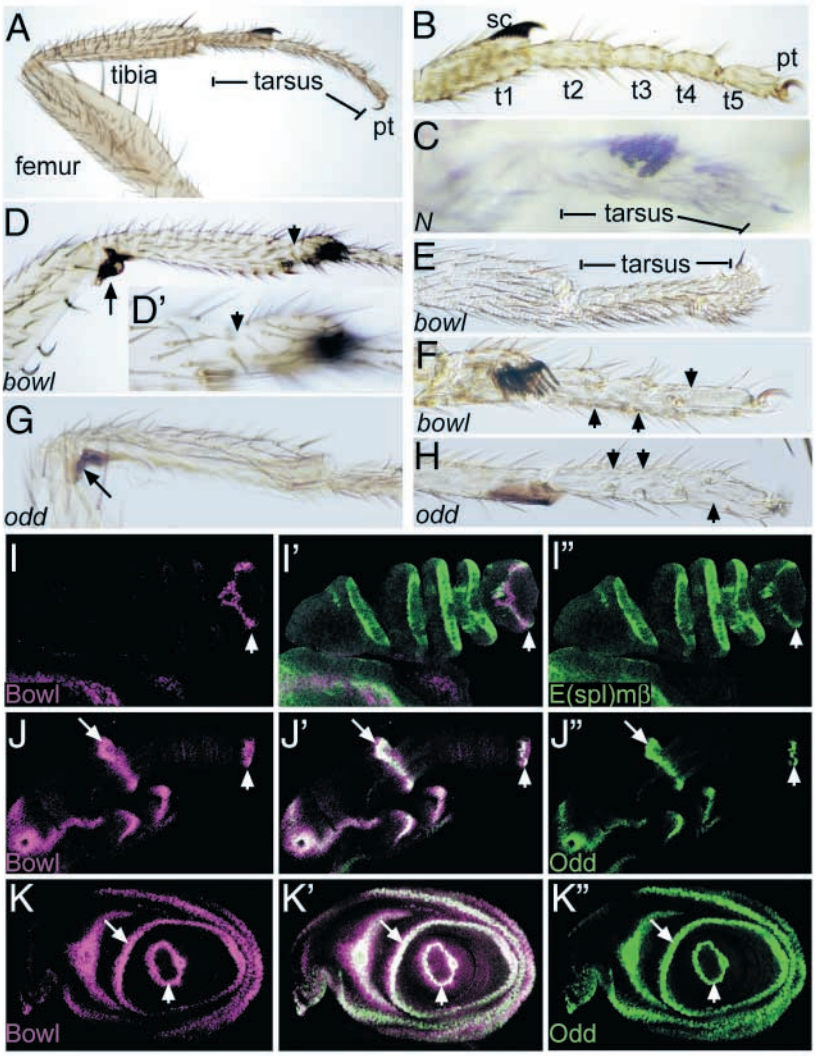

Fig. 1. The *bowl* and *odd* genes give similar phenotypes to *Notch* when mutated but are expressed at only a subset of Notch-dependent boundaries. (A,B) Organization of segments in wild-type prothoracic leg. The femur, tibia, tarsomeres (t1-t5), pretarsus (pt) and sex comb (sc) are indicated. (C) *Notch* mutations result in fusion and truncation of tarsomeres (N^{ts}/Y prothoracic leg, t1-t5 segments are fused). (D-H) Legs containing clones of cells mutant for *bowl* ($bowl^{2}$; D-F) or *odd* (odd^{5}; G-H) result in similar defects to *Notch*. (D,G) Melanotic tissue at femur/tibia joint (arrow) and (D,D′) fused tibia/t1 (arrowhead; inset D′ shows higher magnification), reduced t1 and aberrant sex-combs. (E) Severe fusion and truncation of tarsus when *bowl* clones occupy most of the distal leg. (F,H) Single *bowl* (F) or *odd* (H) clone affecting t1-t4 in prothoracic leg (marked with *forked*, similar results were obtained with *yellow*), causing partial fusions in t2-t5 (arrowheads) and associated truncation. (I-I″) Bowl (magenta) is expressed with *E(spl)mβ-lacZ* (green) at the t5/pretarsus boundary (arrowhead) but not at other sites within the tarsus. (J-K″) Bowl protein (magenta) and *odd-lacZ* expression (green) in everting pupal (J-J″) and third instar (K-K″) leg discs. Expression occurs at intersegment/joint boundaries (tibia/t1, arrows; t5/pretarsus, arrowheads), where the two genes are coexpressed (J′,K′; regions of overlap appear white). Expression of Bowl appears slightly broader than *odd-lacZ*.

present at all the proximal joints (coxa/femur, femur/tibia, tibia/t1) and at a distal site, the t5/pretarsal boundary (Fig. 1J,K; the latter has not previously been documented as a site of Notch activity, although it clearly expresses *E(spl)mβ* and gives rise to an articulated joint). In summary, therefore, Bowl and Odd are present at a subset of the segmental boundaries where Notch is active in the developing leg. These correspond to the boundaries between 'true' segments and not to those between tarsomeres.

bowl is regulated by Notch in the developing leg

Expression of the Notch ligands is a key step in regulating Notch activity in the developing leg (Mishra et al., 2001; Rauskolb, 2001). To investigate the relationship between Bowl and Notch activity, we first compared the timing and distribution of Bowl expression with that of Serrate and Delta, which both regulate Notch activity in the leg disc (Mishra et al., 2001; Rauskolb, 2001). By monitoring expression from early third instar, we found that the evolution of Bowl/*odd-lacZ* expression closely parallels that of the Notch ligands (Fig. 3A-D). The only significant discrepancy appears late in the third instar, when Serrate and Delta are detected at intertarsomere boundaries but Bowl and *odd-lacZ* are not (Fig. 3C,C′ and data not shown). Before that stage, Bowl/Odd expression occurs distal to each domain of Delta that is established. For example, the central t5/pretarsal ring of Bowl appears at ~86 hours (Fig. 3A-C) and correlates with the appearance of Delta in the tarsus and a transient expression of Serrate on the distal, pretarsal, side (Fig. 3A-C and data not shown) (Rauskolb, 2001).

We then tested more directly whether Bowl accumulation at segment boundaries depends on Notch activity, by generating clones of *Notch* mutant cells in the disc epithelium. In all cases in which Notch clones crossed between t5 and the pretarsus, the ring of nuclear Bowl protein at the boundary was interrupted (15/15; Fig. 3E). The effects at the t1/tibia and tibia/femur boundaries were less clear cut, with some clones showing absence (6/21) or reductions (7/21) in Bowl, whereas others retained apparently wild-type levels (8/21). Many of the last group were small clones (seven cells or less; 5/8). In converse experiments, expression of a constitutively activated form of Notch ($Notch^{icd}$) resulted in ectopic Bowl accumulation at a subset of locations in the disc (Fig. 3F). These broadly correspond to the areas where Bowl is normally detected. Taken together, these data indicate that Bowl is responsive to Notch regulation but that the regulation is limited to a specific time window and/or position. Similar results have been obtained with *odd*, which is only responsive to Notch in selected regions (Rauskolb and Irvine, 1999).

Mutations in *bowl* alter the expression of genes involved in tarsal patterning

Neither Bowl nor Odd appear to be present within the tarsus

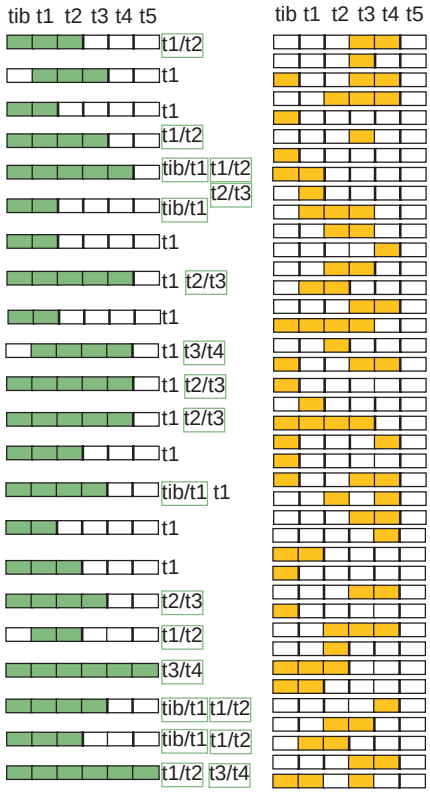

Fig. 2. Phenotypes are only detected in *bowl* clones spanning several tarsomeres. Analysis of 62 legs with *bowl*^{2} clones marked by absence of *y*. Diagrams depict the segments affected in each clone (tibia and tarsomeres t1-t5). 34% of legs analysed had aberrant tarsomeres (green shading indicates extent of clone; specific tarsomeres showing defects are listed, with boxed text indicating segment fusions). 66% of legs containing clones appeared normal (yellow shading indicates extent of clone). Only clones that include t1 (or t5, data not shown) give rise to tarsomere fusions; phenotypes are not detected in clones that only span tarsomere boundaries.

(Fig. 1J-K, Fig. 3C) (Rauskolb and Irvine, 1999), yet mutations in either gene produce defects in this part of the leg (fusion of tarsomeres and growth defects; Fig. 1D-H). There are three models to explain this. (1) Bowl and Odd influence tarsomere segmentation indirectly, by regulating production of a long-range signal. (2) The two genes are expressed at inter-tarsomere joints but at a level too low to be detected. (3) They are involved in an earlier patterning event that influences subsequent tarsal segmentation. We can rule out the first hypothesis, because fusions only occur within tarsal *bowl* clones (Fig. 1D,F; Fig. 2) and the effects on downstream genes are autonomous, as is clearly seen in Fig. 4 (e.g. Fig. 4D). Although it is difficult to rule out the low levels of tarsal expression implied by the second model, our data argue that this is an unlikely explanation for several reasons. First, *bowl* clones that only spanned intertarsomere joints (e.g. t2/t3/t4) appeared normal (Fig. 2). Almost all clones that resulted in observable phenotypes spanned the tibia/t1 boundary and, even within these clones, there was considerable variation in the number of tarsomere joints affected (Fig. 2). This suggests that the effect on tarsomere joints is a secondary consequence of the mutations. Second, in the case of Odd, the pattern of expression detected in the larva persists throughout pupal leg morphogenesis (Mirth and Akam, 2002), ruling out later expression in the tarsomeres. Third, when we produce high levels of *bowl* mRNA using GAL4/UAS system, very little protein is detected in the tarsal domain of late-third-instar/early-pupal legs, suggesting either that the protein is unstable or that the mRNA is poorly translated in this region (e.g. see Fig. 6 and data not shown).

To investigate the third model (that *bowl* has an early patterning function in the leg), we asked two questions. First, we tested whether *E(spl)mβ-CD2* expression in t2-t4 is affected by *bowl* mutations, as predicted if Bowl acts prior to tarsomere boundary formation. As in the adult legs, there is considerable variation between clones, but we observed clear disruptions to *E(spl)mβ-CD2* in three out of nine clones (Fig.

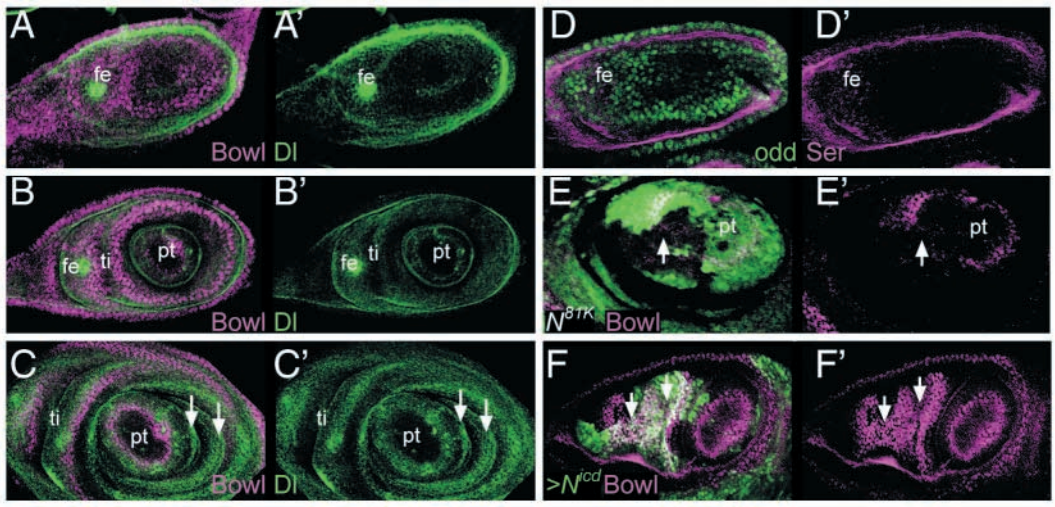

Fig. 3. Expression of Bowl is regulated by Notch activity. (A-C) Bowl expression (magenta, anti-Bowl) correlates with expression of Delta (green, anti-Delta). (A,A′) ~80-84 hour third-instar; Delta expression (green) is found adjacent to Bowl-expressing cells (magenta; presumptive femur fe). (B,B′) ~90 hours, a further domain of Delta (green) is intercalated within the presumptive tibia (ti) and strong expression is seen adjacent to the t5/pretarsal ring of Bowl (pretarsus, pt). (C,C′) ~120 hours, further rings of Delta expression are present within the tarsal region (e.g. arrows C,C′). Bowl is detected at tibia/t1 and t5/pretarsus boundaries, not at intervening sites (arrows, C). (D,D′) 78-80 hours *odd-lacZ* expression (green, anti-β-galactosidase) occurs adjacent to sites of Serrate (magenta, anti-Serrate). Expression of Serrate within presumptive femur is indicated (fe). (E,E′) Bowl expression (magenta) at t5/pretarsus boundary is absent in N^{81K} mutant clones (arrow, marked by absence of GFP). (F,F′) Ectopic Notch activity (green; *act>Gal4/UAS-N*^{icd}) induces ectopic Bowl expression (magenta). Arrows indicate sites with ectopic Bowl (overlap between Nicd and Bowl appears white in F).

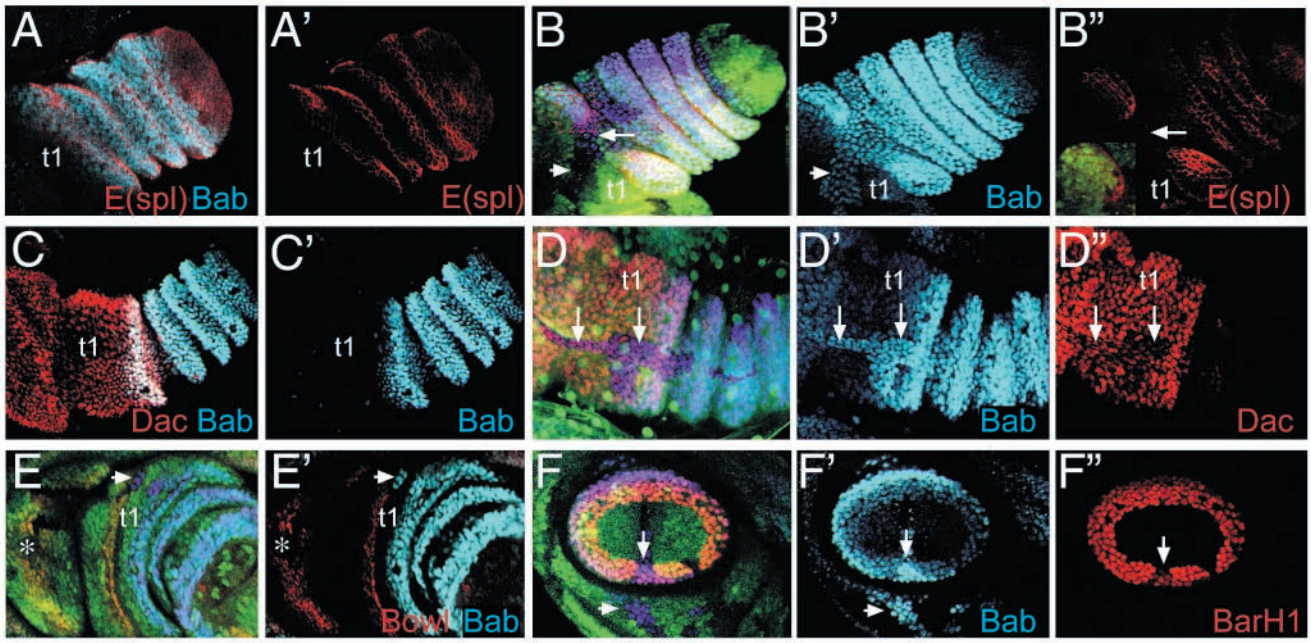

Fig. 4. *bowl* is required for normal patterning of tarsal segments. Expression of Bab2 (anti-Bab2, blue) and *E(spl)mβ-CD2* (anti-CD2, red) in tarsal region of a wild-type pupal leg disc (A) and in a leg disc containing *bowl*^{2} mutant clone (B; mutant cells are marked by absence of GFP, green). Bab expression extends proximally in the mutant cells (arrowhead, B,B′) and *E(spl)mβ-CD2* is disrupted (arrow, B,B″). Inset in B″ (boxed region in B) shows relationship between clone boundaries (green) and *E(spl)mβ-CD2* expression (red); mutant cells at the edge of the clone express *E(spl)mβ-CD2* at wild-type levels. (C,D) Expression of Bab2 (anti-Bab2, cyan) and Dac (red) in wild-type pupal leg disc (C) and in a *bowl*^{2} clone crossing t1 (absence of GFP, arrows, D-D″). The *bowl*^{2} clone results in ectopic Bab2 (blue, D,D′) and decreased Dac (red, D,D″); levels appear higher near the distal edge of the clone because of the fold in the epithelium. (E,E′) *bowl*^{1} clones in proximal tarsus/tibia, Bab2 (blue) is elevated in t1 (arrowhead) and Bowl staining (red) is absent from mutant clones (*). (F-F″) *bowl*^{2} clones (absence of GFP, arrows) in distal tarsus of late third-instar leg disc result in elevated Bab2 (blue) and reduced BarH1 (red). Elevated Bab2 in more proximal clone is also seen (arrowhead).

4B-B″). These defects are confined to the clone but do not strictly follow clone boundaries because some of the mutant cells retain wild-type levels of *E(spl)mβ-CD2* even in the most severe cases (Fig. 4B″, insert). This demonstrates that *bowl* function precedes Notch activation at the tarsomere boundaries and supports the hypothesis that its effects on tarsomere boundaries are secondary.

Second, we asked whether mutations in *bowl* alter the expression of genes involved in the initial regionalization of the leg. Several genes have been identified that confer distinct regional identities and are expressed in broad domains within the leg disc. These include *dachshund* (*dac*), which is expressed in more proximal regions including t1 (Mardon et al., 1994; Lecuit and Cohen, 1997), the two genes of the *bric-a-brac* (*bab*) complex (*bab1* and *bab2*), which are expressed in the presumptive tarsal region (Godt et al., 1993; Couderc et al., 2002; Galindo et al., 2002), and two *Bar* genes that are expressed in distal tarsal segments t4 and t5 (*BarH1* and *BarH2*) (Kojima et al., 2000). We find that expression of all three types of regional genes is affected by mutations in *bowl*. In late-third-instar/early-pupal leg discs, Bab2 expression normally extends to the proximal edge of t1 (Fig. 4A). In *bowl* clones, ectopic Bab2 is detected in proximal parts of t1 and the levels in t2 are also altered (Fig. 4B,D,E). Conversely, when these clones also encompass the distal part of Dac domain, there is a reduction in Dac (Fig. 4C,D). Mutant clones that lie at the distal side of the tarsal domain again show derepression of Bab2 (in distal t5, where Bab2 expression is low or absent), and this is coupled with a reduction in the levels of BarH1 (Fig. 4F). In all cases, the effects are autonomous to the clone and precisely follow clone boundaries.

We therefore conclude that Bowl regulates the expression of patterning genes, promoting development of the proximal (t1/t2) (Fig. 4B,D,E) and distal (t5) extremities of the tarsus (Fig. 4F). Thus, *bowl* mutations lead to an expansion of a 'central tarsal fate' that is characterized by uniform Bab2 and decreased BarH1 and Dac. This disruption in tarsal patterning would in turn affect the expression of Notch ligands in the tarsus and hence lead to defects in tarsomere joints, as seen in the disruption of *E(spl)mβ-CD2* (Fig. 4B). However, there is still a discrepancy between the apparent function of Bowl and its site of expression: Bowl is necessary to inhibit/lower Bab2 expression in t1/t2 and t5, but is not present in these regions in late-third-instar/early-pupal discs. Nevertheless, the effects of *bowl* mutations on Bab2 and Dac are strictly cell autonomous (Fig. 4B,D-F). These observations can be reconciled if Bowl (and likewise Odd) is expressed within the cells that give rise to t1/t2 and t5 at early stages when the domains of the regional genes (*bab*, *dac* and *Bar*) are first established. This expression must subsequently disappear from these regions and become restricted to the boundaries of the tarsus.

Early *bowl*/*odd* expression

If Bowl and Odd are regulating tarsomere segmentation via effects on regional genes like *bab2*, they should be expressed at the boundaries of the Bab2 domain during early stages. At

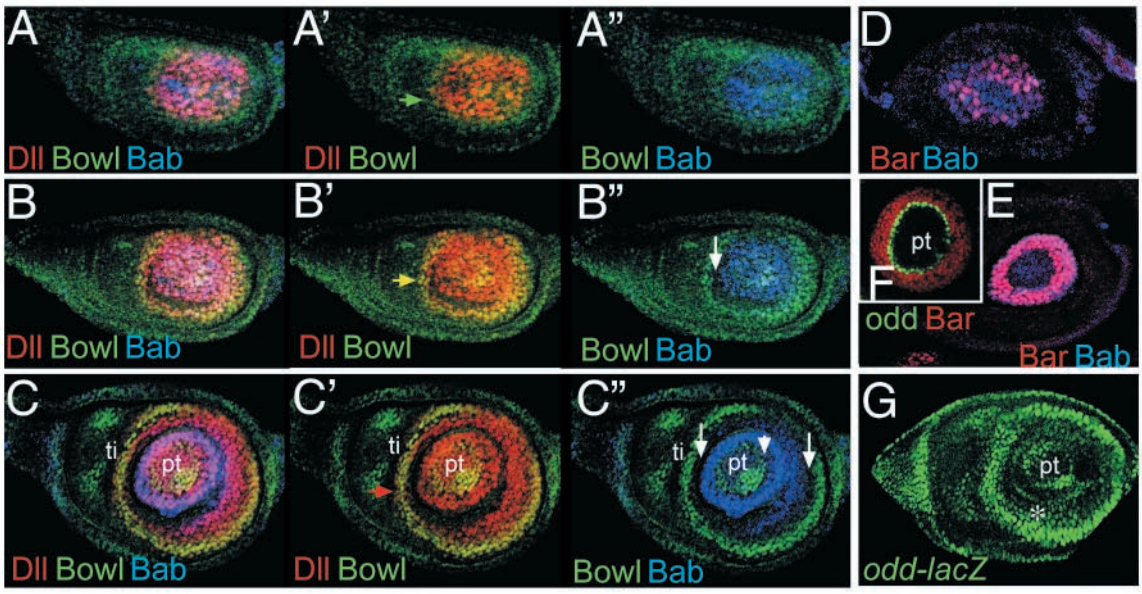

Fig. 5. Expression of Bowl relative to gene products involved in distal leg patterning. (A-C) Expression of Bowl (green, anti-Bowl), Bab2 (blue, anti-Bab2) and Dll (red, *Dll-lacZ*^{01092}) in wild-type leg discs. (A-A″) At ~80 hours, Bowl- and Bab2-expressing cells abut at the edge of the Dll domain. Most Bowl-expressing cells are adjacent to the domain of *Dll-lacZ* (green arrow, A′) (B,B′) At ~86 hours, Bowl-expressing cells are within the *Dll-lacZ* domain (yellow arrow, B′) and a gap (arrow) is appearing between Bab2 and Bowl expressing cells. (C,C′) At 96 hours, *Dll-lacZ*-expressing cells extend more proximally than the Bowl tibia/tarsus ring (red arrow, C′). Bowl-expressing cells no longer abut Bab2 expression domain (C″; arrows, t1/t2; arrowhead, t5). (D-F) Expression of BarH1(red, anti-BarH1) Bab2 (blue) and *odd-lacZ* (green). (D) At 76-80 hours BarH1 expression overlaps most of the Bab2 domain. (E) By 90 hours, Bab2 expression extends more proximally and has disappeared from the central/distal region. (F) At 120 hours, *odd-lacZ* expression abuts BarH1. (G) At 96 hours, *odd-lacZ* expression persists within the presumptive tarsus (e.g. *).

~76-80 hours, both Bowl and Odd are first detected in a 2-3-cell-wide ring that surrounds the Bab2 expressing cells and corresponds to the proximal edge of the Dll domain (Fig. 5A-A″) and the distal edge of the Dac domain (data not shown). Most of these Bab2-expressing cells also express BarH1; on the proximal side only a 1-2-cell-wide ring contains Bab2 and not BarH1 (Fig. 6D). At this stage, therefore, the tarsus consists primarily of one identity, which has Bab2 and BarH1 expression and appears to approximate to t4. This early domain is surrounded by cells expressing Bowl and Odd.

Subsequently, a further ring of Bowl and Odd-expressing cells appears in the centre of the Bab2 domain, at the boundary with the pretarsus, and Bab2 is rapidly lost from within this ring (Fig. 5B-B″). Bab2 is now flanked both proximally and distally by Bowl/Odd. At later stages, gaps appear between Bab2 and the flanking rings of Bowl/Odd (Fig. 5C-C″). These gaps expand and, at the same time, Bab2 expression becomes more graded, with decreasing levels at the edges of its expression domain. This is most marked in the proximal (t2) direction, and is even more evident with Bab1 than with Bab2 (Couderc et al., 2002). Dac expression also extends distally beyond the proximal ring of Bowl/Odd (data not shown) so that it occupies t1 and a small part of t2 by the time the leg disc everts. As a consequence, a series of distinct territories is established within the tarsus by late third instar. Bowl/Odd mark the extreme (tibia/t1 and t5/pretarsal) boundaries of the tarsus and Bab2 expression spans t2-t5 with the peak of its expression in t3/t4.

Both the expression patterns and the phenotypes suggest that cells within the t1/t2/t3 and t5 regions of the tarsal domain contain Bowl/Odd at 76-86 hours. We propose that Bowl/Odd expression is gradually lost from the tarsal cells as they proliferate, giving rise to a temporal gradient of Bowl/Odd (prolonged expression in t1, shorter period of expression in t3/t2). If this is the case, expression from the *odd-lacZ* line might be visible within t1/t2/t3 because of the endurance of β-galactosidase. Indeed, in 96-hour-old *odd-lacZ* discs, we detect β-galactosidase at lower levels within many cells of the tarsus (Fig. 5G). We cannot definitively show a temporal gradient by this method, but the expression of *odd-lacZ* is most persistent close to the final domain of Bowl and Odd, consistent with this model.

Ectopic bowl causes expansion of proximal and distal tarsal fates

Because *bowl* mutations result in expansion of 'central tarsal' (t3/t4) fates, we anticipated that persistent expression of Bowl within the tarsus would have the converse effect, expanding proximal (t1/t2) and distal (t5) fates. To test this we used GAL4 driver lines to direct expression of *UAS-bowl* within the tarsus, scoring phenotypes in the adult male pro-thoracic legs, using the sex comb as a marker of t1 (Fig. 5A-D). Expression of UAS-*bowl* throughout the tarsal region (Dll-Gal4) gave rise to legs with expanded t1 fates manifest in the ectopic sex-combs on distal tarsal segments. In the more strongly expressing lines, the tarsus became severely distorted and carried sex-comb bristles throughout its length (Fig. 6C,D). Even with more restricted production of Bowl (e.g. *klumpfuss-GAL4*) (Klein and Campos-Ortega, 1997), similar transformations occurred, with ectopic sex-comb bristles present in t2 and t3 (Fig. 6B).

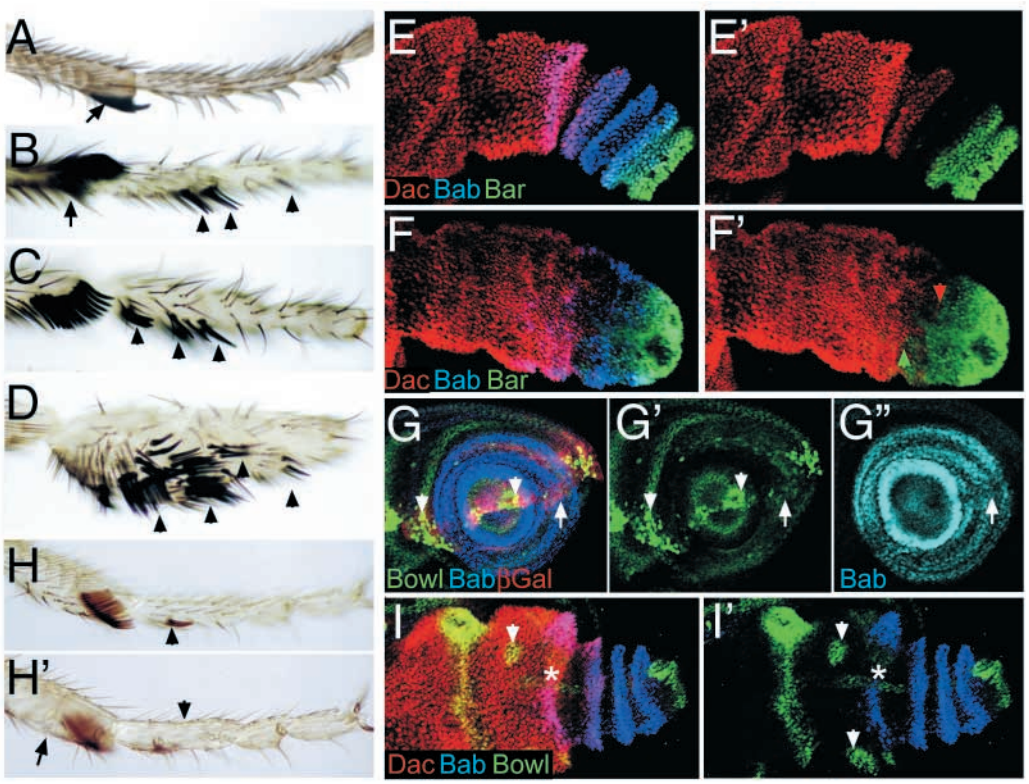

Fig. 6. Ectopic *bowl* expression causes expansion of proximal and distal tarsal fates. (A-D) Expression of *bowl* within distal leg leads to fusion of segments and transformation to more proximal t1 fates as indicated by ectopic sex combs (e.g. arrowheads). (A) Wild-type prothoracic leg; arrow marks the sex comb in t1. (B-D) Prothoracic legs from lines with different levels of *bowl* expression: (B) weak, *klumpfuss-Gal4*^{G410}*/UAS-bowl[6.1]*; (C) intermediate, *Dll-Gal4*^{em212}*/UAS-bowl[9.1]*; (D) strong, *Dll-Gal4*^{em212}*/UAS-bowl[1.1]*. Higher levels of Bowl lead to more severe phenotypes: in (D), the tarsus is completely fused with multiple ectopic sex combs. Domain of *Dll* expression is seen in Fig. 4A-C; *klumpfuss-Gal4*^{G410} is in patches and rings within the tarsal region (Klein and Campos-Ortega, 1997). (E,F) Expression of Dac (red), Bab2 (blue) and BarH1 (green) in pupal legs from wild-type (E) and *Dll-Gal4*^{em212}*/UAS-bowl[9.1]* (F). Ectopic Bowl results in decreased Bab2 and expansion of Dac (red arrowhead) and BarH1 (green arrowhead). (G-G″) Levels of ectopic Bowl protein (green) are more variable than those of β-galactosidase (red), even though both are driven by *ptc-Gal4*. (120 hour leg discs from *Ptc-Gal4*$^{559.1}$*/UAS-bowl[1.1], UAS-lacZ*). Only low levels of Bowl are detected within the tarsus or pretarsus (arrowheads). Expression of *bowl* mRNA is more uniform and similar to *lacZ* (data not shown). (H,H′) Prothoracic leg from *ss*^{a}*/ss*^{114} has ectopic sex comb on t2 (H, arrowhead), ectopic joint in t1 (H′, arrow) and fusion of t2-t3 (H′, arrowhead). (I,I′) In *ss*^{a}*/ss*^{114} pupal leg discs, ectopic patches of Bowl (green) are seen in t1 and t2 (arrowheads). Dac (red) is unaffected but Bab (blue) is decreased in places (*).

To determine whether expansion of t1 fates occurs at the expense of 'central tarsal' fates, we assayed the effects of ectopic Bowl on Bab, Dac and BarH1. In leg discs from *Dll-GAL4/UAS-bowl*, levels of Bab2 were strongly reduced and more patchy than in the wild type, consistent with central tarsal identity being compromised (Fig. 6E,F′). Conversely, the domains of Dac and BarH1 were extended so that they were almost contiguous in the middle of the tarsus (Fig. 6F,F′), demonstrating the expansion of t1 and t5 fates. Ectopic expression of Bowl in a more restricted domain (e.g. with *Ptc-Gal4*) also reduced Bab (Fig. 5G-G″) specifically within the domain of ectopic expression. The inhibition of *bab2* by Bowl fits with the phenotypes of *bab/bab2* loss-of-function alleles, which are similar to that of ectopic Bowl (ectopic sex combs on distal segments) (Godt et al., 1993; Couderc et al., 2002). In analysing the levels of Bowl produced by the directed misexpression, we noted that high levels of protein only accumulated close to the normal sites of expression. Elsewhere, such as within the tarsus, protein levels remain low and patchy (Fig. 6G′), even though mRNA levels are fairly uniform throughout the domain of misexpression (data not shown). Despite the low levels of protein, we still see inhibition of Bab within the tarsus (Fig. 6G″).

Further support for the role of bowl in patterning the proximal tarsus comes from analysing *spineless* mutant legs. This gene is essential for antenna development but is also expressed transiently in the tarsus of the early third-instar leg (Duncan et al., 1998). The phenotype observed in weak *spineless* mutants (*ss*^{a}*/ss*^{114}; Fig. 5H,H′) resembles that of ectopic Bowl (with ectopic sex combs in t2 and an ectopic joint within t1), and we find that the domain of Bowl expression remains broader in *spineless* larval discs and that ectopic patches of Bowl persist in t1 and t2 of early pupal discs (Fig. 6I,I′). Sometimes, the ectopic Bowl forms a discrete ring within t1 that corresponds to the site of the ectopic joint. Persistent Bowl therefore alters P/D patterning, promoting t1-like fates and, in some cases, resulting in an ectopic tibia/t1-like joint. These data suggest that *spineless* is involved in keeping Bowl absent from in the tarsus. In agreement with this, ectopic Spineless results in loss of Bowl (data not shown), although these conditions also result in transformation to antenna fates, complicating the interpretations.

Discussion

P/D patterning in the *Drosophila* limb involves the subdivision of the primordium into concentric regions through the expression of 'gap' genes, whose expression is initiated in response to Dpp and Wg (Lecuit and Cohen, 1997). Subsequent pattern builds on this initial subdivision. Here, we have shown that the genes *bowl* and *odd* are involved in a novel aspect of this process that elaborates the pattern within the

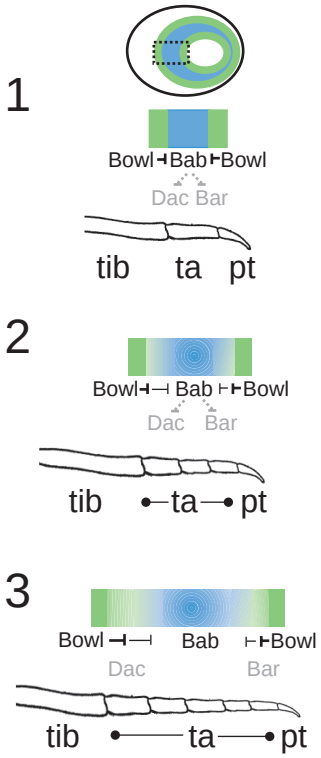

Fig. 7. Model of the relationship between Bowl and Bab2 expression domains and limb patterning. (1) Early stage of tarsal development: a leg imaginal disc with tarsal domains of Bab2 (blue) and Bowl (green). Dotted line outlines region shown below and in subsequent stages. Within this region (rectangle), Bab2 expression (blue) is uniform and flanked by Bowl (green). Bowl inhibits Bab2. Bab2 (or another target of Bowl) prevents upregulation of Dac and BarH1 (dashed grey lines). Below is shown the hypothetical distal leg structures correlating with this stage of expression (Ti, tibia; ta, tarsus; pt, pretarsus), the tarsus consists of a single segment. Arthropods with an unsegmented tarsus are predicted to arrest distal limb patterning at this stage. (2) Middle stage of tarsal development. The length of the tarsal territory (rectangle) has increased. Bab2 expression (blue) is now induced in a larger domain as Bowl (green) decays from the central region (top). Hypothetical distal leg structures correlating with this stage of expression, the tarsus consists of three segments. Arthropods with intermediate numbers of tarsal segments are predicted to arrest distal limb patterning at this stage (bottom). (3) Late stage of tarsal patterning. The length of the tarsal territory (rectangle) has further increased. Bab2 expression (blue) has reached its full extent; as Bowl (green) decays further, Bab2 can no longer be induced and Dac and BarH1 are upregulated in t1 and t5, respectively (top). Distal leg structures correlating with this stage of expression; the tarsus consists of five segments (bottom).

tarsus to generate the correct number and structural diversity of the tarsomeres. Mutations in *bowl* or *odd* cause cells at the proximal and distal positions in the tarsal region to acquire fates of more centrally placed cells, giving rise to truncated or fused tarsomeres. Conversely, ectopic Bowl leads to a transformation of central fates to more proximal or distal fates, again causing distortions and truncations of the tarsus. The changes in fate are manifest in the expression patterns of genes such as *bab1* and *bab2*, which are normally present at the highest levels in t3/t4 tarsomeres and at lower levels in t2 and t5 (Godt et al., 1993; Couderc et al., 2002). Absence of *bowl* leads to elevated Bab2 levels in t2 or t5 and to expression in proximal regions (t1), where *bab2* is normally silent. One notable feature of Bab1/Bab2 expression is that it is modulated into rings of higher and lower expression (Godt et al., 1993). This modulation is also partially lost in *bowl* mutant clones (and in *Dac* mutants) (Chu et al., 2002), arguing that it is intimately associated with the elaboration of patterning.

Previous studies have shown that *bab1/bab2* expression is promoted by Dll and that its proximal and distal limits are dependent on Dac proximally and on epidermal-growth-factor-receptor signalling distally (Campbell and Tomlinson, 1998; Campbell, 2002; Couderc et al., 2002; Galindo et al., 2002). We propose that these activities not only define the initial domain of *bab1*/*bab2* expression but also indirectly regulate the production of Bowl and Odd through their effects on Notch-ligand expression (Rauskolb, 2001). Bowl is then necessary to fine tune *bab2* expression so that its levels are low or absent in the extremities of the tarsus, allowing these to adopt t1 and t5 characteristics (Fig. 7). If one of the factors responsible for positively regulating *bab1/bab2* expression was present transiently, its decay would also contribute to the gradation in Bab2 expression and could explain why Bab2 is not turned on in the t1 cells that have lost Bowl at late stages.

The effects of Bowl and Odd on tarsal development were initially difficult to reconcile with their expression. In late stages of limb development (late L3/early pupal), the proteins are only present at sites of Notch activity outside the tarsus, not within the tarsus, even though the most obvious phenotypes are tarsomere fusions. All of the sites of expression are precursors for the ‘true’ joints (those with tendon attachments and direct muscle control) (Snodgrass, 1935), suggesting that Bowl/Odd could have a primary role in the establishment of joints and that the regulation of tarsal patterning has been acquired secondarily. We propose that effects on patterning occur because the proximal and distal parts of the tarsus are formed by cells that synthesize Bowl/Odd at an earlier stage and that the levels of Bowl/Odd determine the extent of tarsal gene expression (Fig. 7). When the tarsus is first defined by the expression of *bab*, Bowl/Odd directly flank this domain. As the tarsus expands, Bowl and Odd are only retained at the boundary and are lost from the intervening cells; as a consequence, *bab2* is derepressed. In this way, cells closest to the initial domain of Bab2 expression would contain Bowl/Odd for the least time and therefore have higher levels of Bab2 than those closer to the tibial boundary. A similar relationship between expression and phenotype has been seen with *drumstick* (*drm*; a gene related to *bowl* and *odd* that is required for hindgut morphogenesis) (Green et al., 2002). At late embryonic stages, *drm* expression is only detected in the most anterior cells of the small intestine, even though it influences cell behaviour along the whole length of the intestine. By tracing earlier phases of expression, Green et al. were able to show that *drm* is transiently expressed more broadly and gradually becomes restricted to the anterior hindgut boundary (Green et al., 2002), which is similar to what we observed for *odd-lacZ* expression in the leg. It is possible that these similarities in *drm*, *odd* and *bowl* regulation reflect a common underlying mechanism conserved between hindgut and leg morphogenesis.

Notch activation appears to be one key factor in promoting the accumulation of Bowl and Odd at the tarsal boundaries (Fig. 3) (see Rauskolb, 2001), but some data indicate that other factors are required and that the regulation might be indirect. First, Bowl and Odd can only be induced at a subset of the locations where Notch is active, so Notch alone is not sufficient. Second, although all Notch clones at the t5/tibia boundary result in a loss of Bowl protein, not all clones at the more proximal boundaries have a phenotype. Because the smaller clones tend to have the least effect on Bowl, Notch appears to initiate but not to maintain Bowl expression at these locations. Third, although regulation of *odd* can be fully explained by its effects on transcription, Bowl might be subject to post-transcriptional regulation. When we drive

expression of *bowl* mRNA through the leg (using GAL4 drivers), we detect at best low levels of Bowl protein within the tarsus, suggesting that the translation or the stability of the protein are regulated. Candidates to participate in Odd and Bowl regulation include Spineless (Fig. 6) and Lines, a protein that acts antagonistically to Bowl and Drm in hindgut morphogenesis (Iwaki et al., 2001; Green et al., 2002). Although the combined actions of Notch and these factors might explain the initial expression of Bowl and Odd, the mechanism that maintains their expression specifically at the boundaries of the tarsus is unclear. This aspect of regulation is crucial for the diversification of the tarsomeres and, if our model is correct, would be linked to proliferation. Our predictions are that tarsal cells should show a bias in their patterns of proliferation, as is the case in more proximal regions of the leg (Weigmann and Cohen, 1999), and that the progeny of Bowl-expressing cells should occupy the t1/t2 and t5 tarsal segments. We have not yet been able specifically to monitor the proliferation pattern and fate of Bowl-expressing cells to test these predictions.

One extrapolation from our proposed model for tarsal development in *Drosophila* is that the basal or ancestral state consisted of a single tarsal segment, specified by uniform levels of Bab and directly flanked by sites of Bowl expression prefiguring the tarsal/tibial and tarsal/pretarsal joints. This is in agreement with the phylogenetic evidence, which points towards the ancestral arthropod limb having an unsegmented tarsus (as remains the case for many modern arthropods, including some insects) (Snodgrass, 1935). Furthermore, there is considerable variation in the extent of tarsal subdivision, with most insects having between two and five tarsomeres (some arachnids have further subdivisions; Snodgrass, 1935). These differences in pattern could be explained by differences in either the duration or the rate of proliferation during the crucial phase when *bowl/odd* influence *bab2* patterning. Although mutations in *Notch* or *bowl/odd* affect the extent of tarsal proliferation, as do mutations in *spineless* and *bab2*, none of these activities alone is sufficient to cause an increased length of the tarsus (although ectopic Notch activity can give ectopic outgrowth) (Rauskolb and Irvine, 1999). Further investigation of how these factors combine to coordinate tarsal patterning and proliferation should help us to unravel the mechanism underlying the diversification of arthropod limb structure. Furthermore, as modifications of *bab2* expression are correlated with diversification of pigmentation and trichome patterns in *Drosophila* species (Gompel and Carroll, 2003), the possibility that *bab2* expression is intrinsic to diversification of tarsal patterning suggests that changes in the regulation of a single gene could contribute to the diversification of many different morphological traits.

Note added in proof

A related paper by Hao et al. discussing the expression and function of bowl-related genes in the *Drosophila* leg is currently in press (Hao et al., 2003).

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