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Regulation of gene expression in the distal region of the *Drosophila* leg by the Hox11 homolog, C15

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Abstract

The distal region of the *Drosophila* leg, the tarsus, is divided into five segments (ta I–V) and terminates in the pretarsus, which is characterized by a pair of claws. Several homeobox genes are expressed in distinct regions of the tarsus, including *aristaless* (*al*) and *lim1* in the pretarsus, *Bar* (*B*) in ta IV and V, and *apterous* (*ap*) in ta IV. This pattern is governed by regulatory interactions between these genes; for example, Al and B are mutually antagonistic resulting in exclusion of *B* expression from the pretarsus. Although Al is necessary, it is not sufficient to repress *B*, indicating another factor is required. Here, this factor is identified as the product of the *C15* gene, which is another homeodomain protein, a homolog of the human Hox11 oncogene. *C15* is expressed in the same cells as *al* and, together, C15 and Al appear to directly repress *B*. C15/Al also act indirectly to repress *ap* in ta V, i.e., in surrounding cells. To do this, C15/Al autonomously repress expression of the gene encoding the Notch ligand Delta (Dl) in the pretarsus, restricting Dl to ta V and creating a Dl+/Dl− border at the interface between ta V and the pretarsus. This results in upregulation of Notch signaling, which induces expression of the *bowl* gene, the product of which represses *ap*.

Keywords: *Drosophila* leg; *C15*; *Aristaless*; Imaginal disc; Hox11; Bowl; Notch; Delta

Introduction

The role of morphogen gradients in regulating spatial patterns of differentiation in developing tissues is supported by an increasing body of experimental data. Gradients of secreted signaling polypeptides can be visualized in developing tissues and target genes have been identified whose expression is differentially sensitive to the intracellular activity of signaling pathways regulated by these polypeptides (reviewed in Tabata and Takei, 2004). However, the final pattern of expression of these targets usually requires further refinement, often by regulatory interactions between the targets themselves, in particular, direct cross-repressive interactions when the targets encode transcription factors.

Mutual repression results in sharp boundaries between expression domains; such boundaries are difficult to establish simply by differential threshold responses to graded information, which usually result in overlapping domains. Establishing sharp boundaries is often essential to the subsequent generation of precise patterns of cell differentiation. For example, in the vertebrate neural tube, a gradient of Sonic Hedgehog protein activates or represses the expression of several homeobox genes, such as *Nkx2.2* and *Pax6*, but their final pattern of expression is dependent upon mutual repression resulting in sharp boundaries of expression between targets (reviewed in Jessell, 2000). This establishes non-overlapping domains of homeobox gene expression along the dorsoventral axis of the neural tube that is translated into the differentiation of specific neuronal subtypes at precise positions along this axis. Another example of this phenomenon can be found in the early *Drosophila* embryo, where gradients of the transcription factors Bicoid, Hunchback, and Caudal establish the initial expression domains of different gap genes at distinct positions along the anteroposterior axis of the embryo. However, their final expression pattern is dependent upon asymmetric cross-repression between adjacent gap gene

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products (Jaeger et al., 2004; Rivera-Pomar and Jackle, 1996).

Here, we investigate another example of this phenomenon in the developing tarsus of the *Drosophila* leg, the distal-most region of this appendage. Patterning along the proximodistal (P/D) axis of the tarsus is controlled by a distal-to-proximal gradient of EGF-receptor (EGFR) signaling activity, established by a source of ligands in the center of the leg imaginal disc, which corresponds to the presumptive tip of the adult appendage (Campbell, 2002; Galindo et al., 2002; Kojima, 2004). The adult tarsus is divided into five segments (ta I to ta V, from proximal to distal) and terminates in the pretarsus that is characterized by a pair of claws (Fig. 1G). High levels of EGFR activity are required for development of the claws, while progressively lower levels are needed for development of more proximal segments (Campbell, 2002). Similarly, high levels are required to activate expression of the distal-most gene *aristaless* (*al*), which is required for development of the claws and is expressed in the very center of the leg disc (Campbell and Tomlinson, 1998; Campbell et al., 1993; Schneitz et al., 1993), while lower levels are sufficient to activate more proximally expressed genes, such as *Bar* (*B*) (Kojima et al., 2000).

If *B* expression was regulated only through activation by EGFR signaling, it would be expressed throughout the central region of the disc, but it is, in fact, excluded from the cells in the center of the disc that express *al*, and consequently is expressed as a ring surrounding *al*, with no overlap (Kojima et al., 2000). In late third instar discs, this ring corresponds to ta IV and V. Both *al* and *B* encode for homeodomain containing transcription factors (Campbell et al., 1993; Higashijima et al., 1992; Kojima et al., 1991; Schneitz et al., 1993) and previous studies have demonstrated that *al* and *B* are mutually antagonistic (Kojima et al., 2000) so that Al is required to repress *B*, while B can repress *al*, thus accounting for the sharp boundary between their expression domains and the exclusion of *B* expression from the center of the disc. However, although loss of *al* results in expansion of the *B* domain into the center, ectopic expression of *al* does not repress *B* (Kojima et al., 2000), indicating that, although Al is required for repression of *B*, it is not sufficient and at least one additional factor must be required. Another homeobox gene, *lim1*, is also expressed in the same cells as *al*, but as *lim1* mutants are much weaker than those of *al* (Lilly et al., 1999; Pueyo et al., 2000; Tsuji et al., 2000), it does not appear to encode for this missing factor.

Here, this missing factor is identified as the product of the *C15* gene, which is also a homeodomain protein, a homolog of the Hox11 protooncogene of humans (Dear and Rabbitts, 1994; Reim et al., 2003). *C15* is expressed in the same cells as *al*, and legs from *C15* mutants have an identical phenotype to those from *al* mutants. Data are presented to support the proposal that a combination of C15 and Al is required to repress *B* directly. It is also shown that, as well as directly repressing *B*, C15/Al can also repress expression of genes such as *apterous* (*ap*) non-autonomously, in surrounding cells. This is achieved through upregulation of Notch signaling in surrounding cells, paradoxically through direct repression of the gene encoding the Notch ligand Delta (Dl) in the pretarsus by C15/Al.

Materials and methods

Fly strains

Flies carrying the following existing alleles or transgenes were used: al^{ice}, al^{130} (*In(2L)*al^{130}), *Df(3R)e-19*, *Df(3R)e-BS2*, *ap-lacZ* (ap^{rK568}), *al-lacZ* (al^{X21}), *UAS-al* ($al^{lScerUAS.cSa}$), *UAS-lim1* (*Lim1*^{Scer}UAS.cTb), $lim1^{R12.4}$, *UAS-Egfr.lambdatop*, $Egfr^{tsla}$, $Egfr^{f24}$, *ptc-Gal4* (*Scer\GAL4*$^{ptc\text{-}559.1}$), *tub > CD2 > Gal4* (*Scer\GAL4*$^{ScerFRT.RnorCD2.\alpha Tub84B}$), $bowl^{1}$, *UAS-GFP* (*Avic\GFP*$^{ScerUAS.T:HsapMYC,T:SV40nls2}$), *hs-flp* (*P{hsFLP}22*), *FRT82B* (*P{ry[+t7.2] = neoFRT}82B*), *Ubi-GFP* (*P{Ubi-GFP(S65T)nls}3R*), *FRT40A* (*P{ry[+t7.2] = neoFRT}40A*),

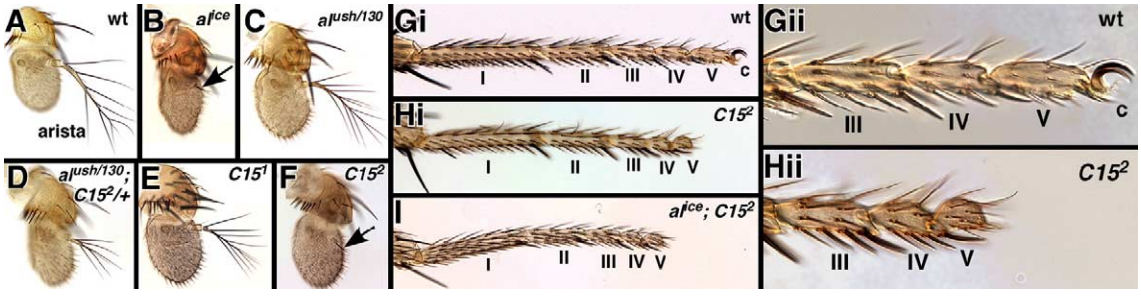

Fig. 1. Screen for enhancers of *al* and phenotype of *C15* mutants. (A–F) Adult antennae. (A) Wild-type antenna showing the terminal structure, the arista. (B) In strong *al* mutants, the arista is reduced to a vestige (arrowed). (C) $al^{ush/130}$ is a weak mutant in which the arista is almost full length. (D) The phenotype of $al^{ush/130}$ can be dominantly enhanced by mutations in several genes, including *C15*. (E, F, H) Antennae and legs from homozygous *C15* mutants; the *al* gene is wild-type. (E) $C15^{1}$ is a weak mutant with a slightly shorter arista (compare to A). (F) $C15^{2}$ is a strong mutant with antennal and leg phenotypes indistinguishable from *al*. (G–I) Distal region of adult legs. (G) The tarsus from wild-type legs is divided into five segments (I–V) and terminates in a pair of claws (c). (H) In strong *C15* mutants the claws are absent and tarsal segments IV and V are reduced. (I) Legs from al^{ice}; $C15^{2}$ double mutants are indistinguishable from those of single mutants.

hs-GFP (*Avic\GFP*$^{hs.T:HsapMYC}$), *M(2)201*, *M(3)95A* (*Rp S3*^{Plac92}), *Bar-lacZ* (*B-H2*^{P058}), *FRT101* (*P{FRT (whs)}101*), *N*^{XK11}, *UAS-N*^{intra} (*N*$^{intra.GS.ScerUAS}$), *Dl*^{RevF10}, *UAS-Dl* (*P{UAS-Dl.H}MH1*), *Dl-lacZ* (Dl^{05151}), *dpp-Gal4* (*P{GAL4-dpp.blk1}40C.6*). Unless indicated otherwise in parentheses, all genotypes are as denoted in Flybase (http://flybase.bio.indiana.edu), where more information on each can be found. *al*^{ush} was a gift from Pascal Heitzler and is the weakest *al* allele having an almost wild-type phenotype as a homozygote. The '*Egfr*^{ts}' genotype refers to the allelic combination *Egfr*^{tsla}/*Egfr*^{f24} (Kumar et al., 1998); mutant larvae were shifted to the restrictive temperature during the third instar. UAS-C15 flies were generated by standard transgenic methods using the pUAST vector (Brand and Perrimon, 1993) containing a *C15* cDNA (amplified by RT-PCR from imaginal discs).

Screen to uncover dominant enhancers of the al phenotype

al^{ush} males were mutagenized with EMS by standard procedure (Grigliatti, 1986) and crossed to *al*^{130}/*CyO* females. Progeny with reduced aristae were selected. Enhancers of the *al* mutant phenotype were mapped using deficiencies. The enhancer characterized in this paper, *C15*, was originally uncovered by the deficiencies *Df(3R)e-19* and *Df(3R)e-BS2*, placing it in the interval 93C3–93F. The location of this enhancer was supported by the demonstration that these deficiencies also enhanced the *al*$^{ush/130}$ phenotype. All available mutations in genes included in the region 93C3–93F complemented *C15* mutations.

Clonal analysis and ectopic expression

Homozygous mutant clones were generated in imaginal discs by *hs-flp*/FRT-induced mitotic recombination (Xu and Rubin, 1993). Clones were generated in the second or early third instar of larvae with the following genotypes: *hs-flp*; *FRT82B C15*^{3}/*FRT82B Ubi-GFP M(3)95A* (and similarly for *Dl*^{RevF10}); *hs-flp*; *bowl*^{1} *FRT40A*/*M(2)201 hs-GFP FRT40A*; *N*^{XF11} *FRT101*/*hs-flp hs-GFP FRT101*.

Clones were identified by loss of GFP expression

Clones ectopically expressing *C15*, *al, lim1*, *N*^{intra}, and *Dl* were generated using a combination of the UAS/Gal4 system and the FLPout technique (Pignoni and Zipursky, 1997; Struhl and Basler, 1993) in larvae with the following genotypes: *hs-flp UAS-GFP*; *UAS-C15*; *Tub>CD2>Gal4* (and similarly for *UAS-al*, *UAS-lim1*, *UAS-N*^{intra}, *UAS-Dl*).

Larvae were given a 34°C heat shock for 1 h in early third instar; clones were identified by GFP expression.

Immunostaining and analysis of adult legs

Dissection and staining of imaginal discs was carried out by standard techniques. The following antibodies were used: anti-Al (rat; 1:1,000) (Campbell et al., 1993); anti-C15 (rat, 1:1000); anti-B (rabbit, 1:5) (Higashijima et al., 1992); anti-βgal (rabbit, Cappell, 1:2000), anti-Lim1 (guinea pig, 1:1000) (Lilly et al., 1999); anti-Ap (guinea pig, 1:1000) (Fernandez-Funez et al., 1998); anti-Bowl (rabbit, 1:200) (de Celis Ibeas and Bray, 2003); and anti-Nub (mouse, 1:5 (Ng et al., 1995). To generate the C15 antibody, a GST fusion protein containing the C-terminal region of C15, corresponding to residues 262–307, was injected into a rat. Secondary antibodies were from Jackson immunochemicals (Cy5 conjugates, at 1:200) and Molecular Probes (Alexa 488 and Alexa 568 conjugates at 1:500). Legs from adult flies were mounted in GMM (Lawrence and Johnston, 1986).

Results

A genetic screen for modifiers of the aristaless (al) mutant phenotype

To uncover genes encoding for proteins that are required for Al activity or lie upstream or downstream, a screen was devised to identify genes, that when mutated, could dominantly modify the phenotype of a weak *al* mutant. In null *al* mutants, the arista, the terminal portion of the antenna, is lost almost completely, although a vestige remains (Figs. 1A, B). However, in a weak allelic combination, *al*$^{ush/130}$, the arista is almost full length (Fig. 1C). Random mutagenesis yielded several mutants that dominantly reduced the size of the arista in *al*$^{ush/130}$ flies (Fig. 1D). One of these genetic enhancers was characterized as a mutation in the EGF-receptor (*Egfr*^{Eal43}), which has been shown to lie upstream of *al* (Campbell, 2002; Galindo et al., 2002), indicating that the screen could be successful in its goals. In this paper, one more of these dominant enhancers will be characterized and, as described below, it was shown to correspond to the *C15* gene, which encodes for a homolog of the vertebrate homeodomain protein, Hox11 (Dear and Rabbitts, 1994; Reim et al., 2003). In fact, three alleles of *C15* were identified in the screen, *C15*^{1}, *C15*^{2} and *C15*^{3}, and are the first mutations identified in this gene.

C15 mutant legs and antennae are identical to those from al mutants

When the *al* mutant alleles were separated from the *C15* alleles, homozygotes of all three *C15* alleles survived to adulthood, although two, *C15*^{2} and *C15*^{3}, died soon after emerging. Examination of these adults revealed that their legs and antennae had identical phenotypes to that of *al*, i.e., the aristae and the claws were either reduced or completely lost (Figs. 1E–H). In the weakest mutant, *C15*^{1}, the aristae were reduced (Fig. 1E) but the claws were normal (not shown); this is similar to weak *al* mutants such as *al*^{1} (Stern

and Bridges, 1926). In the stronger two mutants, $C15^2$ and $C15^3$, the arista was almost completely eliminated apart from a very small vestige and the structures found at the tip of the leg (claws, pulvilli, and empodium) were completely eliminated (Figs. 1F, H). In addition, although there are still five tarsal segments, ta IV and V were reduced to about half their normal size (Figs. 1G, H). All these phenotypes are identical to those of null or very strong alleles of *al* (Campbell and Tomlinson, 1998), but much stronger than that of null *lim1* mutants, which often possess a claw (Pueyo et al., 2000; Tsuji et al., 2000).

Molecular characterization of the C15 mutations

These mutants were shown to correspond to *C15* as follows. First, they were placed in the interval 93C3–93F by deficiency mapping; this region includes *C15*. All other available mutations in this region complemented the *C15* alleles. Second, in situ hybridization showed that *C15* was expressed in the center of the leg and antennal discs (not shown), i.e., in cells giving rise to the regions affected in the mutant adults. Finally, sequencing of each of the mutants identified a single base change in *C15* that, in $C15^1$, results in substitution of a conserved residue N-terminal to the homeodomain (H175Q), and, in both $C15^2$ and $C15^3$, results in a stop codon truncating the protein at residues 170 and 137, respectively. Both truncations occur before the homeodomain, suggesting that these two are probably null alleles.

C15 is expressed in the same cells as al and lim1, but is regulated independently by EGFR signaling

An antibody raised against C15 revealed that it was expressed in exactly the same cells as Al and Lim1 in the center of leg discs (Fig. 2A), so that its expression domain abutted that of B (Fig. 3A), which is expressed in ta IV and V (there are actually two *B* genes, *H1* and *H2*, which are co-expressed) (Higashijima et al., 1992; Kojima et al., 2000). To determine if *C15* lies downstream of Al or vice versa, their expression was examined in discs from the reciprocal mutant. Each was still expressed, but its expression domain was significantly reduced (Figs. 2B, C). In contrast, Lim1 expression was lost completely in both *C15* and *al* mutant discs (Figs. 2B, C) (Pueyo et al., 2000; Tsuji et al., 2000). In addition, although there is some variation, the expression domains of C15 and Al were only mildly reduced in *lim1* mutants (Figs. 2D, E).

If C15 is not downstream of the other homeobox genes expressed in the center of the disc, it must be activated by another mechanism. *al* expression is induced by EGFR signaling (Campbell, 2002; Galindo et al., 2002), raising the possibility that C15 may also be under EGFR control. This was confirmed by loss and gain of function experiments, as follows. First, C15 expression was lost in discs from an $Egfr^{ts}$ mutant grown at the restrictive temperature (29.1°C) at which *al* expression is lost (Fig. 2F). Second, misexpression of a constitutively active form of the EGFR (UAS-Egfr.lambdatop) resulted in ectopic expression of C15 (Fig.

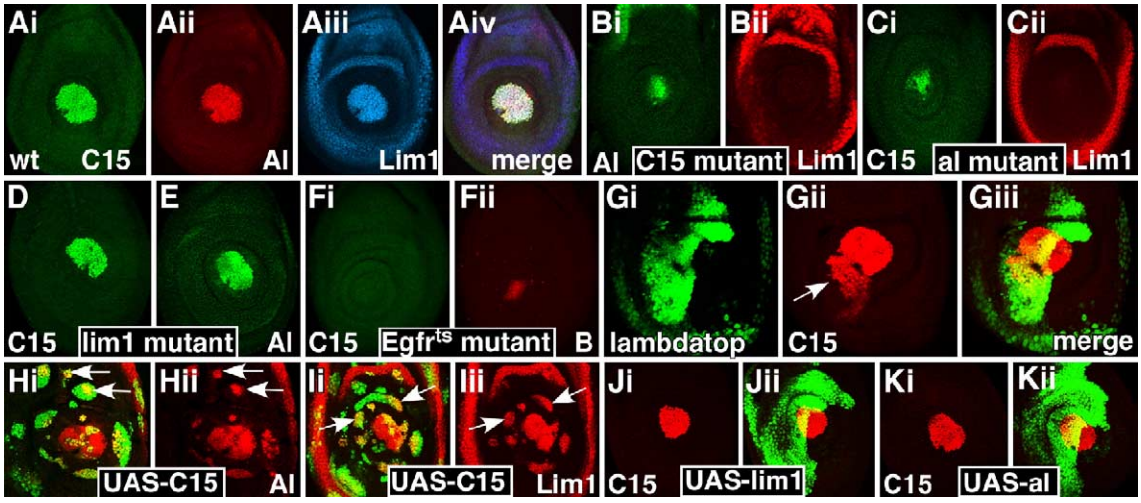

Fig. 2. Expression of C15, Al, and Lim1 in late third instar leg imaginal discs. (A) These three homeodomain proteins are expressed in the same cells in the center of the disc (C15, green; Al, red; Lim1, blue). There is also extensive Lim1 expression more proximally. (B) Al (green) is still expressed, but in a smaller domain in the center of $C15^2$ discs, but Lim1 (red) is not, although it is still expressed more proximally. (C) Similarly, C15 (green) is expressed in the center of al^{ice} discs, but Lim1 (red) is not. (D and E) Both C15 and Al are expressed in the center of $lim1^{R12.4}$ discs, although their domains are slightly reduced in size. (F) C15 (green) expression is lost in $Egfr^{ts}$ mutants grown at 29.1°C during the third instar (i), but B (red) expression can still be detected in the center (ii). (G) Misexpression of a constitutively active form of the EGFR, lambdatop, using dpp-Gal4 (expression identified with UAS-GFP in green) results in ectopic expression of C15 (red) in the ventral region (arrow). (H and I) Leg discs containing clones of cells ectopically expressing *C15* (green, made using the FLPout system and tub>CD2>Gal4; clones marked with UAS-GFP). Ectopic *C15* can induce ectopic expression of Al and Lim1 (red; arrows), although not in all of the cells ectopically expressing *C15*. (J and K) Ectopic expression of *al* or *lim1* using dpp-Gal4 (expression identified with UAS-GFP in green) does not induce expression of C15 (red). (C15, Al and Lim1 expression was detected using antibodies, apart from Aii, where a *lac-Z* enhancer trap in *al* was used.)

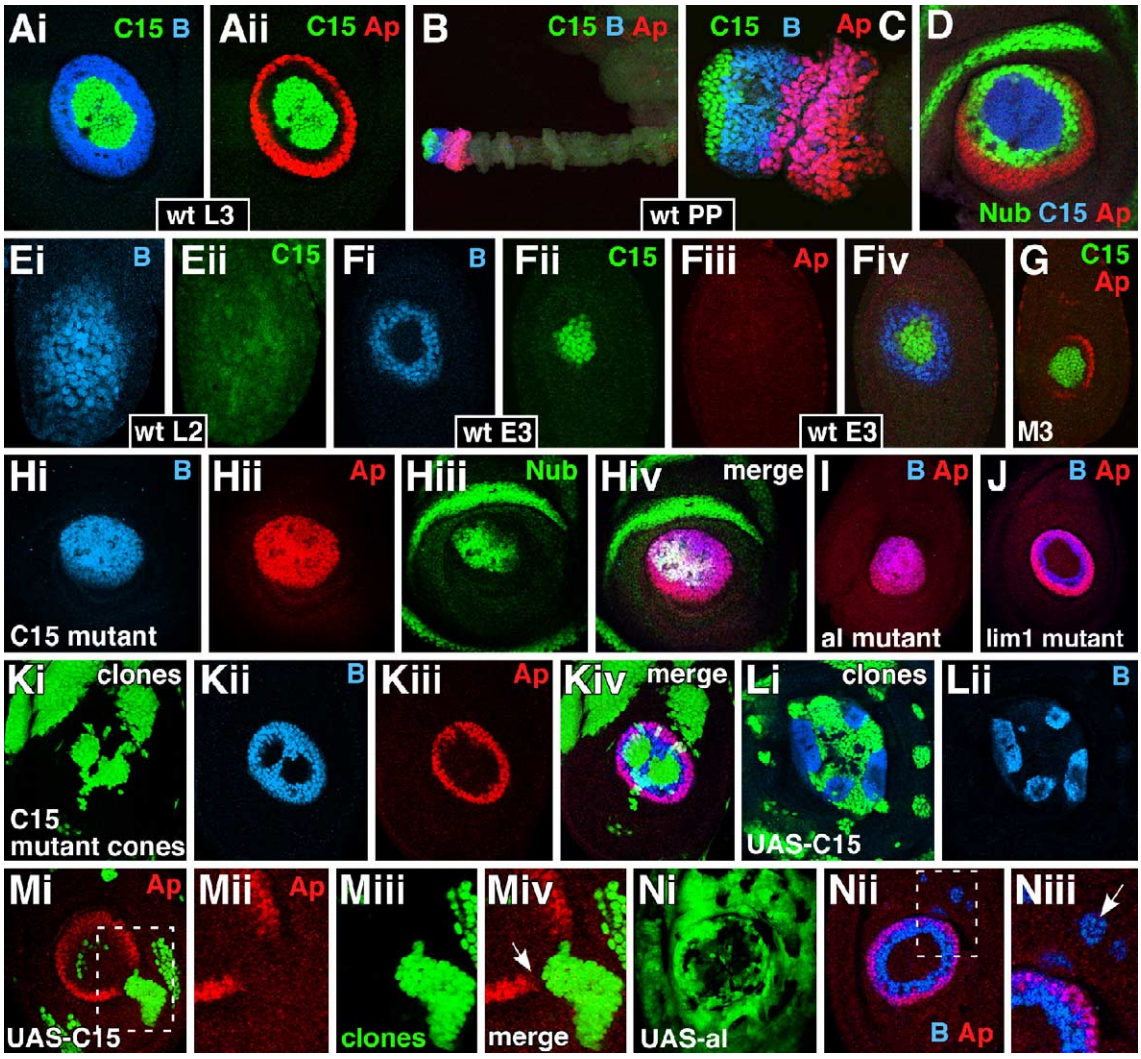

Fig. 3. Regulation of *B* and *ap* expression by C15. (A) Center of wild-type leg disc from late third instar. B (blue) is expressed in the cells surrounding C15 (green) with no overlap, while there is a gap between C15 and Ap (red). (B and C) Partially everted leg from prepupa (PP). C15 (green), B (blue), and Ap (red) are expressed at the tip of the tarsus, C15 at the very tip, B in tarsal segments IV and V, and Ap overlapping B in segment IV. (D) Nub is expressed in a ring surrounding C15, but in a narrower domain than B because it does not overlap with Ap (the Ap ring is out of focus in the dorsal region of this disc). (E) Late second instar (L2) leg disc. No C15 (green) expression can be detected (ii) but B (blue) is expressed throughout the center (i). (F) Early third instar (E3) disc. C15 (green) is now expressed and B (blue) is lost from these cells. Ap (red) is not expressed yet. (G) Mid-third instar (M3) leg disc in which Ap (red) expression can just be detected. Note, even at this stage there is a gap between Ap and C15 (green). (H) $C15^{2}$ mutant leg disc. Expression of B (blue), Ap (red), and Nub (green) expression now extends into the center. B and Ap are both expressed in exactly the same cells, but Nub is expressed in a smaller domain in the center. (I) al^{ice} mutant leg disc. B (blue) and Ap (red) expression is identical to that in *C15* mutants (H). (J) $lim1^{R12.4}$ mutant leg disc showing that B (blue) and Ap (red) are absent from the center. (K) Leg disc containing *C15* mutant clones (black, identified by the loss of a ubiquitous GFP marker). B (blue) is upregulated in the *C15* mutant cells in the center (ii), but Ap (red) is not (iii). (L–N) Leg disc containing clones of cells ectopically expressing *C15* or *al* (green, made using the FLPout system and tub>CD2>Gal4; clones marked with UAS-GFP). Ectopic expression of *C15* results in autonomous loss of B expression (blue; L), but non-autonomous loss of Ap expression (red, arrow; M, ii–iv are a magnification of the box in i). However, ectopic expression of *al* does not result in repression of B (blue, N) or Ap (red, N), and, in fact, can induce some ectopic expression of B in a small percentage of cells (arrow, iii is a magnification of the box in ii), but not Ap. (C15, B, Lim1 and Ap expression was detected using antibodies apart from Aii, B, C, Ei, Fiii, G, where *lac-Z* enhancer traps in *B* and *ap* were used.)

2G); similar to other EGFR targets, this ectopic expression was restricted to the ventral region (Campbell, 2002).

To further investigate any regulatory interactions between C15, Al, and Lim1, each was misexpressed in the leg and expression of the other two examined. This was achieved initially with a UAS-C15 line and by generating Gal4 expressing clones using the FLPout technique and Tub-Gal4; the clones were monitored with UAS-GFP. This revealed that ectopic *C15* could, in fact, induce ectopic expression of both Al and Lim1, although this was somewhat random with Al and Lim1 being expressed only in some cells ectopically expressing *C15* (Figs. 2H, I). As shown below, ectopic *C15* can also repress *B* (Fig. 3L) and loss of *B* has previously been shown to result in expansion of the Al expression domains (Tsuji et al., 2000), but only in the cells immediately surrounding their normal domains.

Repression of *B* does not appear to account for the ectopic Al and Lim1 expression induced by *C15*, because, Al and Lim1 can be induced some distance from their endogenous domains (Figs. 2H, I). In contrast, misexpression of *al* or *lim1* in Tub-Gal4 clones had no effect on expression of the other genes (not shown). Previous reports indicated that driving higher levels of *lim1* could induce ectopic expression of *al* (Tsuji et al., 2000) and we confirmed this using dpp-Gal4 (not shown). However, there was no ectopic C15 in the UAS-lim1; dpp-Gal4 discs (Fig. 2J). Similarly, driving higher levels of *al* with dpp-Gal4 did not induce ectopic expression of *C15* (Fig. 2K).

Therefore, although Al is still expressed in *C15* mutants, and vice versa (Figs. 2B, C), indicating that both are probably activated independently by EGFR signaling, C15 can induce expression of *al* and *lim1*. This may act as a feedback mechanism to ensure all three are expressed in the same cells. As expression of Lim1 is completely lost in the center of discs from *C15* and *al* mutants (Figs. 2B, C) (Pueyo et al., 2000; Tsuji et al., 2000), it may simply be a direct target of either or both and may not be directly activated by EGFR signaling.

As *al* is still expressed, albeit in a much smaller domain, in *C15* mutants and *C15* is still expressed in *al* mutants (Figs. 2B, C), it appeared possible that each may play an additional, redundant role, in patterning the leg. This was ruled out by examining al^{ice}, $C15^{2}$ double mutants (both alleles are either null or very close to being null), which had legs and antennae that are indistinguishable from either single mutant (Fig. 1I) (Campbell and Tomlinson, 1998); indicating that, in the absence of the other, neither Al nor C15 provides any function during leg development.

C15 acts directly to repress B in the center of the leg

In late third instar discs, B is expressed in the cells immediately surrounding C15 (Fig. 3A), as has already been described for Al (Kojima et al., 2000). In partially everted discs, this corresponded to C15 at the very tip and B in ta V and IV (Figs. 3B, C). The *nubbin* (*nub*) gene is expressed in ta V (Fig. 3D) (Rauskolb and Irvine, 1999) overlapping with B in ta V but not in IV. With antibody staining, B and C15 could first be detected in very early third instar and both appeared to be expressed at the same time. B is already excluded from the center at this stage (Fig. 4F). However, using a lac-Z enhancer trap in *B*, which is more sensitive than antibody staining, β-gal expression was detected even earlier in late second instars. At this stage, when no C15 could be detected, β-gal expression was found throughout the center of the disc (Fig. 3E). Slightly later when C15 becomes detectable, β-gal was excluded from the center. Al was first detected at approximately the same time as C15 (not shown).

The loss of B and Nub expression from the center of the disc can be explained by repression by Al and C15. Loss of *al* has been shown to result in expansion of *B* expression into the center of the disc (Tsuji et al., 2000), indicating Al is required to repress *B* in this position. Not surprisingly, *C15* null mutant discs had the same phenotype (Fig. 3H). The diameter of the domain of B is slightly smaller than the diameter of the B ring in wild-type discs. Nub expression is also found in the center of *C15* mutant discs, but in a smaller domain than B (Fig. 3H), indicating that there are still distinct differences between ta IV and V in *C15* mutants.

Repression of *B* by C15 is strictly autonomous, as shown in discs containing *C15* mutant clones, where B expression expanded into all the cells in the center that lost C15 (Fig. 3K). In addition, ectopic expression of *C15* resulted in autonomous repression of B (Fig. 3L). Curiously, although we confirmed previous studies that showed ectopic *al* cannot repress *B* (Kojima et al., 2000), we also found that it could actually induce ectopic expression of B in more proximal regions of the disc (Fig. 3N).

C15 acts indirectly to repress ap in the center of the leg

B expression is absent from the center of the leg, specifically from the cells expressing Al and C15 (Fig. 3Ai).

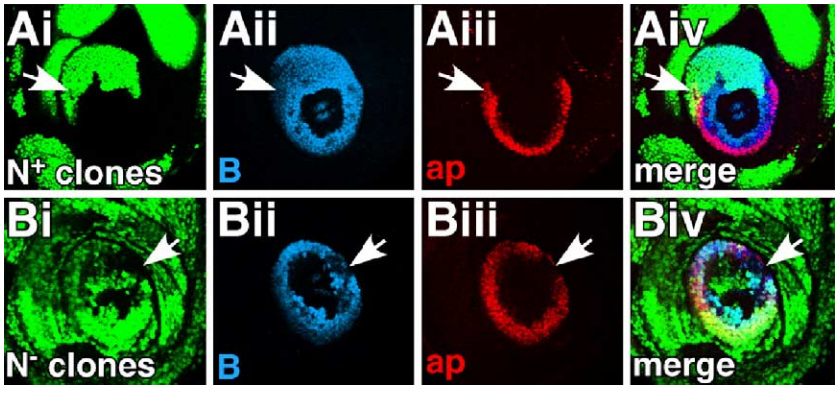

Fig. 4. Repression of *ap* by Notch signaling. (A) Leg disc containing clones of cells ectopically expressing N^{intra} (green, made using the FLPout system and tub>CD2>Gal4; clones marked with UAS-GFP). Ap expression is lost and B expression is expanded in the central N^{intra} clone, although at the edge of the clone some Ap expression remains (arrow). (B) Leg disc containing homozygous mutant clones of *Notch* (black, identified by the loss of the ubiquitous GFP marker). Expression of B and Ap is lost or reduced in *Notch* mutant clones (arrow).

However, other genes, including *ap* and *bab*, are absent from a more extensive region in the center (Godt et al., 1993; Pueyo et al., 2000), and there is a gap between the C15 expression domain and Ap and Bab (Fig. 3Aii; not shown). Consequently, Ap expression is restricted to presumptive tarsal segment IV, where it overlaps with *B* (Figs. 3A, C) but not with Nub, which is expressed only in ta V (Fig. 3D). It has been suggested that, as well as activating genes such as *al* and *B*, EGFR signaling may directly repress genes in the center of the disc, possibly accounting for the absence of *ap* and *bab* in this location (Campbell, 2002; Galindo et al., 2002). Surprisingly, we discovered that *ap* and *bab* expression, as well as *B*, is regulated by C15/Al. In both *C15* and *al* mutant discs, Ap and Bab expression expanded into the center of the disc (Fig. 3H; not shown). Consequently, in regard to Ap expression, the distal region of the leg adopts a tarsal segment IV-like fate. However, Nub, which is normally only expressed in ta V, is now co-expressed with Ap in the very center, indicating that the distal-most segment in C15 legs has characteristics of both ta IV and V.

In wild-type discs, Ap expression was first detected slightly later than B, Al, or C15 (Figs. 3F, G), but even at this time there was a clear gap between it and C15 (Fig. 3G), indicating that C15/Al acts non-autonomously to repress *ap*. This was supported by two further studies. First, unless there was a complete loss of *C15* in homozygous mutant discs, Ap expression was not derepressed in C15 mutant clones in the center if the clones were not too large (Fig. 3K), indicating surrounding wild-type C15-expressing cells can rescue the mutant tissue. Second, ectopic expression of *C15* resulted in non-autonomous repression of Ap (Fig. 3M).

These results suggest that EGFR signaling represses gene expression in the center of the disc only indirectly through activation of C15/Al. This is also supported by two other observations. First, Al is still expressed in *C15* mutant discs (Fig. 2C), indicating that EGFR signaling levels are still very high in the center of these discs, but *ap* is not repressed (if *ap* was repressed directly by EGFR, its threshold for this would be lower than the threshold for activation of *al* because *ap* is repressed further from the source in the center than *al* is activated). Second, ectopic expression of *C15* results in non-autonomous repression of *ap* (Fig. 3M), but, if this was due to increased EGFR signaling in surrounding cells, then it should result in activation of EGFR targets such as *B* immediately adjacent to the cells expressing C15 (outside of the normal *B* domain), but does not (Fig. 3L). Consequently, it seemed very likely that C15 uses an alternative mechanism to repress *ap*, most likely by upregulation of a signaling pathway in surrounding cells (i.e., ta V).

Notch signaling can repress ap expression

The ability of different signaling pathways to repress *ap* expression was tested and it was discovered that upregulation of the Notch pathway in ta IV (by misexpression of the Notch intracellular domain) resulted in loss of Ap expression (Fig. 4A). Curiously, however, Ap expression was not upregulated in Notch mutant cells, and was, in fact, lost or downregulated (Fig. 4B; the phenotype is somewhat variable), indicating low-level Notch signaling is required for Ap expression, possibly indirectly, because loss of Notch can also lead to downregulation or loss of B expression in ta IV (Fig. 4B) and B is required for expression of *ap* (Kojima et al., 2000).

Bowl can repress ap and is activated non-autonomously by C15

Notch signaling usually represses gene expression indirectly by inducing expression of repressors, so known Notch targets in the distal leg were tested to determine if they were required for repression of *ap*. The best candidate appeared to be the *bowl* gene which encodes a zinc finger transcription factor that is expressed in a ring in the distal leg under the control of Notch signaling and can both activate and repress gene expression (de Celis Ibeas and Bray, 2003; Wang and Coulter, 1996). To investigate if *bowl* is involved in repressing *ap* expression, mutant clones were generated in leg discs. In these clones, cells expressing high levels of Ap now directly abutted those expressing C15, i.e., there was no gap between them (Fig. 5A). Low-level Ap expression could also be detected in clones that extended into the C15 domain, indicating Bowl is also required here but that an additional factor, possibly C15/Al, can partially repress *ap* in this location (if so, C15/Al would be acting autonomously in a similar fashion to repression of *B*). Ectopic *bowl* expression can also repress Ap expression (Fig. 5B). The response to ectopic *bowl* was fairly weak, but it appears that ectopic expression of this gene does not result in high levels of protein expression (de Celis Ibeas and Bray, 2003).

Examination of Bowl and Ap expression in leg discs revealed that there is a gap between their expression domains (Fig. 5C), even at a time when Ap expression was first detected in mid-third instars (Fig. 5D). This could indicate that Bowl acts non-autonomously to repress *ap*. However, the clonal analysis clearly showed that Bowl acts autonomously: any wild-type cells expressing Bowl had no influence on Ap expression in surrounding mutant tissue (Fig. 5A). It is possible that there is low-level Bowl expression in the ‘gap’ that cannot be detected with antibody staining. Another possible explanation is one of timing, and that Bowl was expressed in the cells in the ‘gap’ slightly earlier and that this is sufficient to silence the *ap* gene even before its expression can be detected more proximally. The possibility that *bowl* is expressed transiently in cells was proposed earlier to explain the observation that *bowl* mutant clones have effects in central regions of tarsus, i.e., in regions where

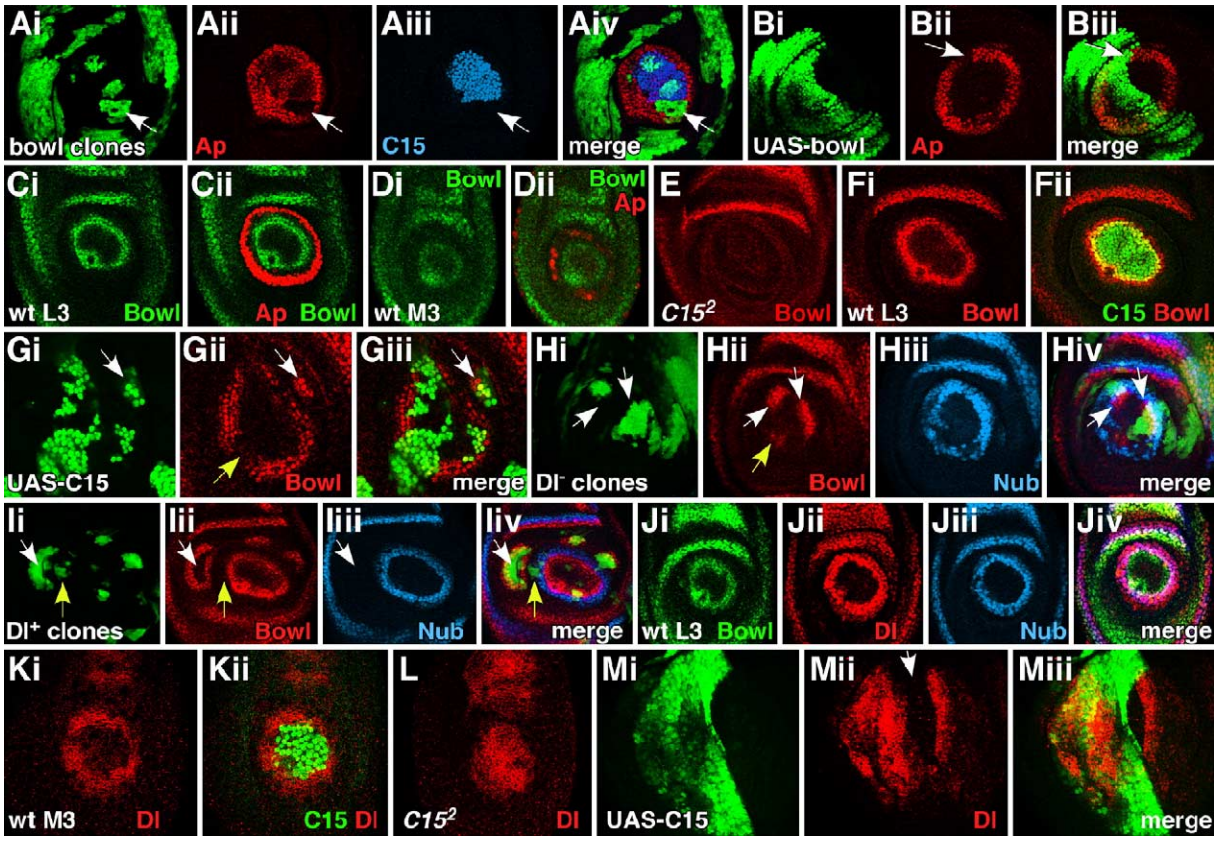

Fig. 5. Repression of *ap* by Bowl and activation of *bowl* expression by C15 and Dl. (A) Leg disc containing homozygous mutant clones of *bowl* (black, identified by the loss of the ubiquitous GFP marker). There is no gap between Ap (red) and C15 (blue) in *bowl* mutant cells. There is also ectopic expression of Ap in the center overlapping with C15, although this is weaker than in the ring surrounding it. Note that *bowl* appears to function autonomously: Ap is repressed in the remaining wild-type cells surrounding the C15 domain (arrowed), but not in any adjacent cells. (B) Ectopic expression of *bowl*, using dpp-Gal4, results in repression of Ap (red), primarily in the dorsal region where the driver (visualized with UAS-GFP in i) is strongest (arrow). (C) In discs from wild-type, late third instars (L3), Bowl (green) is expressed in a ring in the center (and more proximally). However, there is a gap between Bowl and Ap (red). (D) Mid-third instars (M3) at about the time when Ap expression (red) can first be detected. Even at this stage, there appears to be a gap between Ap and Bowl. (E) Bowl expression is lost in the center of $C15^2$ mutant discs. (F) Wild-type disc from late third instar (L3) showing the ring of Bowl (red) is usually 2 cells wide, the inner ring of cells comprising the outermost cells of the C15 domain (green, the overlap being yellow, ii), while the outer ring surrounds C15. (G) Leg disc containing clones of cells ectopically expressing *C15* (using the FLPout system and tub>CD2>Gal4; clones marked with UAS-GFP). Ectopic expression of *C15* can result in both ectopic expression (white arrow) and repression (yellow arrow) of Bowl (red). Some of the ectopic expression is non-autonomous, in cells surrounding GFP positive cells. Repression of *bowl* is explained by C15 repressing expression of Dl (see J, L), as *bowl* expression also requires Dl (see H, I). (H) Leg disc containing *Dl* mutant clones (black, identified by the loss of a ubiquitous GFP marker). Bowl expression is lost in most of the mutant tissue, but is present adjacent to wild-type cells (white arrow; the yellow arrow marks cells expressing Bowl in a circular domain, possibly corresponding to glial cells that is not dependent upon Dl; Hao et al., 2003). Nub is still expressed in *Dl* mutant cells even far from any wild-type cells, although it is irregular. (I) Leg disc containing clones of cells ectopically expressing *Dl* (green, made as in G). Ectopic expression of *Dl* results in ectopic expression of Bowl but not Nub. Ectopic Bowl overlaps with or is adjacent to the cells misexpressing Dl. However, in large clones (white arrow), there is no Bowl expression in the center and it is only expressed in the cells at the edge of the clone and the cells surrounding the clone. Clones located immediately proximal to the endogenous Bowl ring in the center of the leg do not induce ectopic Bowl (yellow arrow). (J) Dl expression (red) is upregulated in a ring corresponding to ta V, overlapping with the ring of Nub (blue) in the center. The Bowl ring (green) in the center partially overlaps with Dl, but the most distal Bowl expressing cells are outside of the Dl domain. (K) In wild-type discs from mid-third instars (M3), there is strong Dl expression (red) in a ring surrounding the C15 domain (green). (L) In $C15^2$ mutants, the Dl expression remains, but is now in the very center of the disc, indicating C15 is involved in repressing it. (M) Misexpression of C15 with dpp-Gal4 (marked by UAS-GFP in green) results in repression of Dl (red), especially in the dorsal region where the driver is the strongest (arrow). Curiously, the lower levels of C15 in the anterior appear to be associated with upregulation of Dl expression. (C15, Bowl, Nub, Ap and Dl expression was detected using antibodies apart from C and D and J where *ap-lacZ* and *Dl-lacZ* lines were used, respectively.)

its expression cannot be detected later (de Celis Ibeas and Bray, 2003).

Bowl is, thus, required to repress *ap* expression in tarsal segment V and this predicted that C15 regulates *bowl* expression. This was confirmed by analysis of *C15* mutant discs, in which Bowl expression in the center is lost, although other, more proximal, domains of expression are normal (Fig. 5E). The ring of Bowl in the distal tarsus is usually just two cells in width with the inner cell overlapping with C15, but the outer cell being outside the C15 domain (Fig. 5F), suggesting C15 can induce *bowl* non-autonomously. This was supported by the ability of cells ectopically expressing *C15* to activate Bowl expression in surrounding cells (Fig. 5G). This ability is fairly limited, but

would be expected because the endogenous C15-expressing cells only appear able to induce *bowl* in their immediate neighbor (resulting in a ring of *bowl* expression in a single row of cells surrounding the C15 domain; Fig. 5F).

Delta activates bowl, but Delta expression is repressed by C15

If Notch signaling induces *bowl* expression and C15 is also required for *bowl* expression, it was predicted that C15 upregulates Notch signaling by regulating the expression of the Notch ligand responsible for activation of *bowl*. Although, both Notch ligands, Delta (Dl) and Serrate are expressed in leg discs, it was discovered that only Dl is required to induce expression of *bowl*. *bowl* expression is lost in homozygous *Dl* mutant clones, although, if positioned appropriately, wild-type cells can rescue *bowl* expression in adjacent cells laterally and distally (Fig. 5H). Curiously, *nub*, which was also thought to be a target of Notch signaling (Rauskolb and Irvine, 1999), is still expressed in *Dl* mutant cells (even far from wild-type cells), albeit in an irregular pattern (it is expressed at normal levels in some cells, but at lower levels or not at all in others; Fig. 5H). Misexpression of Dl can also induce ectopic *bowl* expression both in adjacent cells and in the cells misexpressing Dl. However, in large clones, cells in the center of the clone do not express *bowl*, which is only expressed in the cells at the edge of the clone and in the cells immediately adjacent to the clone (Fig. 5I). Nub was not ectopically expressed following misexpression of Dl; this result contrasts with another report which indicated that it could (Rauskolb and Irvine, 1999). The reasons for these conflicting results are unclear, but could be due to different Gal4 drivers being used. The levels of expression induced by the Tub driver in the experiments reported here (Fig. 5G) are sufficient for activation of *bowl* expression and would have been expected to activate *nub* expression also if it was a simple Dl target.

In wild-type mid-third instar discs, Dl expression is upregulated in ta V (Rauskolb, 2001), overlapping with Nub, but not with C15 (Figs. 5J, K). Distally, it overlaps partially with Bowl, although Bowl is also expressed even more distally (Fig. 5J). Proximally, however, Dl does not appear to induce expression of Bowl, suggesting there is a repressor of Bowl expressed in this location. This is supported by the inability of cells misexpressing Dl in this position (proximal ta V, ta IV) to activate Bowl (Fig. 5I). Although it might be predicted that C15 would induce expression of *Dl*, in fact the opposite was found, and C15 actually represses *Dl* in the center of the disc. In *C15* mutants, Dl expression expands into the center of the disc (Fig. 5L) and misexpression of C15 can repress expression of *Dl* (Fig. 5M). How C15-repression of *Dl* can result in upregulation of Notch signaling in cells in ta V surrounding the pretarsus is discussed below.

Discussion

Direct repression of genes in the center of the leg disc by C15/Al

The center of the leg imaginal disc, the presumptive tip of the leg, is characterized by the co-expression of three homeobox genes, *al*, *lim1*, and, as described here, *C15* (Figs. 2A, 6) (Campbell, 2002; Galindo et al., 2002; Kojima, 2004; Lilly et al., 1999; Pueyo et al., 2000; Schneitz et al., 1993; Tsuji et al., 2000). *al* and *C15* are expressed here because EGFR signaling levels are highest in this location (Figs. 2F, G) (Campbell, 2002; Galindo et al., 2002), while it is unclear if this is also true for *lim1* or if it is just a target of C15 and Al. The center of the leg disc is also characterized by the absence of expression of several genes, including *B* and *ap* (Figs. 3A, 6) (Kojima et al., 2000; Pueyo et al., 2000; Tsuji et al., 2000), which are expressed more proximally but which would be expected to extend into the center because they are also activated by EGFR signaling (Campbell, 2002). Here, we show that both *B* and *ap* are repressed in the center by a combination of C15 and Al but that *B* is repressed by a different mechanism than *ap*, and accounts for the observation that *ap* is absent from a wider domain in the center than *B*.

Neither C15 nor Al is sufficient to repress alone, as shown, for example, in *al* mutant discs where *C15* is still expressed, and in *C15* mutant discs where *al* is still expressed (Figs. 2B, C; although both in smaller domains), but in both mutants B and Ap expression extends into the very center, i.e., they overlap with C15 or Al (Figs. 3H, I). Although Lim1 is co-expressed with C15 and Al, B and Ap are still repressed in *lim1* mutants (Fig. 4J), which also have almost normal expression domains of Al and C15 (Figs. 2D, E). However, there can be minor derepression of *B* in the center of *lim1* mutant discs, suggesting it does have a minor role in augmenting C15 and Al activity, that may account for the defective development of the claws (Pueyo et al., 2000; Tsuji et al., 2000).

B is repressed autonomously by C15/Al (Figs. 4K, L), consistent with one or both of these factors binding directly to cis-regulatory sequences at the *B* locus. There is indirect evidence that Al can bind to these sequences in the absence of C15, because ectopic expression of *al* can occasionally induce ectopic expression of *B* (Fig. 4N). This would imply that Al cannot act as a transcriptional repressor alone, at least for *B*, and that it may recruit C15 for this purpose.

Indirect repression of ap by C15/Al through upregulation of Notch signaling in surrounding cells

Other genes expressed in the developing tarsus, such as *ap* and *bab* (Godt et al., 1993; Pueyo et al., 2000), are also excluded from the very center of the disc, but in these cases, this exclusion zone is larger than for that of *B*, so they are absent from the region fated to form ta V as well as the cells

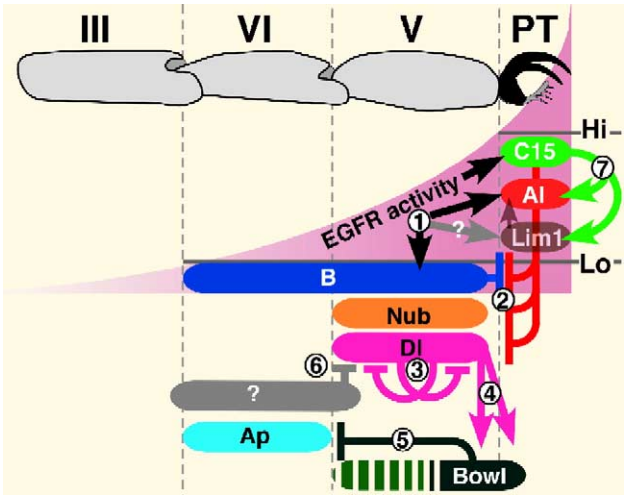

Fig. 6. Model summarizing the genetic interactions that establish patterns of gene expression along the proximodistal (P/D) axis in the distal region of the leg, specifically in tarsal segments IV, V, and the tip of the leg, the pretarsus (PT). The latter is characterized by expression of *C15*, *al*, and *lim1*. *B* is expressed in segments IV and V. *nub* and *Dl* are expressed in segment V. *B*, *nub*, and *Dl* are absent from the PT. *ap* is expressed in segment IV, and is absent from V and the PT. *bowl* is expressed in cells straddling the boundary between ta V and the PT. The sequence of events leading to these patterns is as follows. (1) A gradient of EGFR activity is established along the P/D axis of the tarsus from a source of ligands in the center of the leg disc [Vein and TGF-alpha(s); not shown]. EGFR activates expression of *C15*, *al*, (and possibly *lim1*) above a high signaling threshold (Hi) and *B* (and possibly *nub* and *Dl*) above a lower threshold (Lo), explaining why *B* is expressed more proximally. (2) C15/Al and B are mutually antagonistic resulting in exclusion of *B* from the PT and establishing a sharp boundary between their expression domains. C15/Al also directly repress *nub* and *Dl* excluding them from the PT. C15 and Al act in combination to repress *B* in the cells in which they are expressed. Lim1 plays a minor role in repression of *B*. (3) The Notch ligand, Dl, is expressed in segment V, but cannot signal to other cells in ta V expressing Dl, with the exception, (4), of those that border cells not expressing Dl, i.e., those at the distal edge of ta V that abut the PT. Cells in the PT, that do not express Dl, can also respond to Dl expressed in ta V. Consequently, Notch signaling is upregulated in cells on either side of the boundary between ta V and the PT, and this induces expression of *bowl*. In the absence of C15, Dl expression expands to the tip of the leg so there is no Dl+/Dl− boundary, and no upregulation of Notch signaling and no *bowl* expression. (5) Bowl represses *ap* expression in segment V. The dashed line indicates the discrepancy between where Bowl is known to act genetically and but where its expression cannot be detected. This may correspond to where it was expressed earlier in development but has been lost because, as the disc grows, these cells become situated further from the Dl+/Dl− boundary between ta V and the PT. (6) Dl appears unable to signal proximally, possibly because a repressor (unidentified) of Notch signaling or *bowl* is expressed in this location. (7) C15/Al may also positively regulate expression of *al* and *lim1*, although *al* is still expressed in the absence of C15. Lim1 may positively regulate expression of *al*, but not *C15*.

expressing *C15*/*al* in the very center. Consequently, there is a clear gap between their expression domains and that of *C15*/*al* (Figs. 3A, 6, not shown). However, C15/Al are also required to repress expression of these genes in the center of the leg (Figs. 4H, I) and do this non-autonomously (Fig. 4M), suggesting they regulate the expression or activity of a signaling molecule that leads to upregulation of a signaling pathway in the cells surrounding those expressing C15/Al. This appears to be the Notch pathway because upregulation of this in ta V results in loss of *ap* expression (Fig. 4A).

The majority of our results are consistent with a model in which C15/Al upregulate Notch signaling in surrounding cells in ta V (and those at the edge of the pretarsus) through direct repression of the gene encoding the Notch ligand Dl in the pretarsus (Fig. 6). This results in high levels of Dl expression only in ta V surrounding the pretarsus. Previous studies have shown that if a cell expresses Dl, it is often unresponsive to Dl in adjacent cells (de Celis and Bray, 1997; Doherty et al., 1996; Jacobsen et al., 1998; Micchelli et al., 1997; Panin and Irvine, 1998; Rauskolb and Irvine, 1999). The results presented here on the ability of Dl to induce expression of *bowl* indicate that, in the distal leg, cells expressing D1 in ta V can signal to adjacent Dl− cells in the pretarsus, but also appear to be able to signal to adjacent Dl+ cells in ta V, but only those at the distal edge of the Dl domain, i.e., cells that are also bordering Dl− cells in the pretarsus. Thus, Notch signaling is upregulated in a ring of cells straddling the ta V/pretarsus boundary. The key event that facilitates this is the repression of *Dl* expression in the center of the leg by C15/Al because this creates a Dl+/Dl− border that is essential for Dl to activate Notch (Fig. 6). Notch signaling upregulates expression of *bowl*, which encodes for a transcription factor that appears to directly repress *ap* in ta V.

Support for this model is as follows. Loss of *bowl* results in *ap* expression in cells immediately surrounding C15/Al (Fig. 5A), while ectopic expression of *bowl* can repress *ap* expression (Fig. 5B). *bowl* expression is dependent upon Dl, it being lost in *Dl* mutant clones, apart from mutant cells immediately adjacent to wild-type Dl-expressing cells (Fig. 5H). *bowl* expression can also be induced by clones of cells misexpressing Dl, both in cells adjacent to the clone and cells within the clone, but only those at the edge; cells in the center of large Dl+ clones do not express *bowl* (Fig. 5I). In wild-type discs, Dl expression is upregulated in ta V, while the Bowl expression domain is usually two cells in width with one cell in the pretarsus (overlapping with C15/Al; Fig. 5F) and one in ta V (overlapping with Dl; Fig. 5J). In *C15* mutants, Dl expression extends into the center (Fig. 5L) and in common with large clones ectopically expressing Dl, there is no *bowl* expression in the center (Fig. 5E). The lack of *bowl* expression at the proximal border of the central Dl domain appears to be due to repression of Notch signaling or *bowl* itself by an, as yet, unidentified factor.

There are, however, some inconsistencies in this model. First, upregulation of Notch in ta V does not always repress all of the *ap* expression, in particular at the edge of a clone (Fig. 4A). Second, although clonal analysis shows that Bowl represses *ap* strictly autonomously (Fig. 5A), there is always a gap between cells expressing Bowl and those expressing Ap (Figs. 5C, D). It is possible that the antibody being used to monitor Bowl expression cannot detect lower levels of protein present in the gap. Alternatively, Bowl may only be transiently expressed in the gap. A previous study

also reported a discord between where Bowl protein can be detected and where it is required (de Celis Ibeas and Bray, 2003). Consequently, further studies are required to investigate these possibilities.

Establishing patterns of gene expression in the distal tarsus

This study also addresses more general questions about how signaling gradients can generate expression of mutually antagonistic targets that are activated above different signaling thresholds, such as *B* and *C15*/*al* (Fig. 2F) (Campbell, 2002), with *B* being activated above a lower threshold of EGFR signaling activity than *C15*/*al*. Consider what happens as a gradient of signaling activity is established across a group of cells following expression of a secreted signal. Initially, signaling levels will be low and the low-threshold target should be expressed close to the source, while the high-level target should not be expressed yet. This is supported by observations in the early leg disc where *B* expression can be detected in the center of the leg prior to expression of *C15*/*al* (Fig. 3E).

However, B represses expression of the high-threshold targets *al* and *C15*, the expression of which expands slightly when B function is removed (Kojima et al., 2000; and data not shown), so how are *al* and *C15* ever expressed in cells already expressing B even when signaling levels rise? Expression of high-threshold targets such as *C15*/*al* is probably a balance between one negative and two positive influences: (1) repression by the low-threshold target, B; (2) activation from the signaling pathway, here, the EGFR pathway; and (3) the ability of C15/Al to repress B once they are expressed. Presumably, at high ligand levels, activation by EGFR signaling is sufficient to overcome any repression from B and *C15*/*al* will be expressed even in the presence of B. This is supported by observations here: in *al* mutants, for example, *C15* is still expressed in the very center of the disc where EGFR signaling levels are highest, even though B is co-expressed there (Figs. 2C, 3H, I). However, the size of the *C15* domain in *al* mutants is much smaller than in wild-type discs (Fig. 3C); this may be explained by the apparent inability of C15 to repress B on its own so now there is only a single positive influence, EGFR signaling, disturbing the normal balance in favor of repression by B. Alternatively, the smaller C15 domain in *al* mutants may reflect a reduction in cell proliferation or increase in cell death in the very center following loss of Al.

Function of the C15 homolog, Hox11, in vertebrates

Hox11 is required for development of the spleen in mice (Roberts et al., 1994), while misexpression is associated with specific T-cell leukemias in humans (Dube et al., 1991; Hatano et al., 1991; Kennedy et al., 1991). Consequently, uncovering the mechanisms it uses to regulate gene expression is crucial for understanding these processes, in particular transformation. Like C15, Hox11 appears to be capable of repressing gene expression (Owens et al., 2003). There is, as yet, no evidence that Hox11 interacts with any homologs of Al, but studies on C15 in *Drosophila* may provide further insight into the mechanisms it uses to regulate gene expression.

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