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Delta and Hairy establish a periodic prepattern that positions sensory bristles in *Drosophila* legs

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Abstract

In vertebrates and invertebrates, spatially defined proneural gene expression is an early and essential event in neuronal patterning. In this study, we investigate the mechanisms involved in establishing proneural gene expression in the primordia of a group of small mechanosensory bristles (microchaetae), which on the legs of the *Drosophila* adult are arranged in a series of longitudinal rows along the leg circumference. In prepupal legs, the proneural gene *achaete* (*ac*) is expressed in longitudinal stripes, which comprise the leg microchaete primordia. We have previously shown that periodic *ac* expression is partially established by the prepattern gene, *hairy*, which represses *ac* expression in four of eight interstripe domains. Here, we identify *Delta* (*Dl*), which encodes a Notch (N) ligand, as a second leg prepattern gene. We show that Hairy and Dl function concertedly and nonredundantly to define periodic *ac* expression. We also explore the regulation of periodic *hairy* expression. In prior studies, we have found that expression of two *hairy* stripes along the D/V axis is induced in response to the Hedgehog (Hh), Decapentaplegic (Dpp) and Wingless (Wg) morphogens. Here, we show that expression of two other *hairy* stripes along the orthogonal A/P axis is established through a distinct mechanism which involves uniform activation combined with repressive influences from Dpp and Wg. Our findings allow us to formulate a general model for generation of periodic pattern in the adult leg. This process involves broad and late activation of *ac* expression combined with refinement in response to a prepattern of repression, established by Hairy and Dl, which unfolds progressively during larval and early prepupal stages.
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Keywords: *hairy*; *achaete*; *Delta*; *Notch*; Leg imaginal disc; Prepattern genes

Introduction

The nervous systems of vertebrates and invertebrates are highly ordered structures, which are formed, at least in part, by common developmental mechanisms. An early event in both *Drosophila* and vertebrate neuronal patterning, for example, is a spatially defined proneural gene expression. Proneural genes in *Drosophila* function in the selection of neural precursors from a field of ectodermal cells. In vertebrates, region-specific proneural gene expression is necessary for the temporal and spatial patterning of neuronal differentiation (reviewed by Gomez-Skarmeta et al., 2003).

The function and regulation of proneural gene expression have been intensively investigated in the *Drosophila* peripheral nervous system (PNS). During adult PNS development, expression of two redundant proneural genes, *achaete* (*ac*) and *scute* (*sc*), confers neural competence and marks the positions of future sensory organs. *ac* and *sc* are initially expressed within small groups of cells, called proneural clusters, at specific sites of the adult body wall and limb primordia (Cubas et al., 1991; Romani et al., 1989; Skeath and Carroll, 1991). Expression is later refined, through lateral inhibition mediated by Delta (Dl)/Notch (N) signaling, to one or a few cells of the cluster, which will give rise to sensory organ precursor(s) (SOPs). Elegant studies in the dorsal mesothorax, or notum, have shown that proneural gene expression is regulated by a group of prepattern genes that are expressed in distinct and partially overlapping subdomains of the notal primordium (reviewed by Gomez-Skarmeta et al., 2003). The differential and partially overlapping expression of these prepattern genes defines the

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domains of proneural gene expression through activation and repression.

In this study, we focus on the prepattern genes that control expression of *ac* in the primordia of a group of small mechanosensory bristles, or microchaetae, found on the legs of the second thoracic segment (T2 legs). The T2 leg microchaetae are organized into a series of longitudinal rows precisely positioned around the leg circumference. We have previously shown that, in the tarsus of mid-prepupal legs, *ac* is expressed in longitudinal stripes that comprise the proneural fields from which the microchaete SOPs will be selected. Furthermore, *hairy* (*h*), which encodes a bHLH transcriptional repressor (Ohsako et al., 1994; Van Doren et al., 1994), acts as a prepattern gene that partially establishes periodic expression of *ac* (Orenic et al., 1993). *hairy* is expressed in two pairs of longitudinal stripes, one pair that traverses the dorsal/ventral (D/V) axis (D/V-*hairy*) and another pair that runs along the anterior/poster (A/P) axis (A/P-*hairy*) (Carroll and Whyte, 1989; Orenic et al., 1993). Each *hairy* stripe is expressed between a pair of *ac* stripes, defining four of eight *ac* interstripe domains. In the absence of *hairy* function, *ac* expression expands into the regions normally occupied by Hairy, broadening the microchaete proneural fields and resulting in disorganized bristle rows in the adult. Strikingly, a recent study has shown a similar function for two zebrafish *hairy*-related genes, the *hairy*- and *enhancer of split-related* genes (*her* genes), *her3* and *her9* (Bae et al., 2005). During development of the zebrafish central nervous system, proneural gene expression in longitudinal stripes along the A/P axis of the dorsal ectoderm establishes proneural domains from which primary neurons will form. *her3* and *her9* are expressed in striped domains that are complementary to the proneural stripes, and these *hairy*-related genes function to spatially define proneural gene expression through repression. Furthermore, spatial regulation of *her3* and *her9* in zebrafish (Bae et al., 2005) and *hairy* in *Drosophila* (Hays et al., 1999; Kwon et al., 2004) is controlled in part by the conserved bone morphogenetic protein (BMP)/Decapentaplegic (Dpp) signaling pathway. These observations suggest a conserved prepattern function and at least partial similarity in regulation of *hairy*-related genes during vertebrate and invertebrate neuronal patterning.

The conservation of mechanisms that generate periodic pattern in the *Drosophila* and vertebrate nervous systems suggests that there are important insights to be gained from investigating how the pattern of sensory bristles in the *Drosophila* adult leg is generated. Although we have some understanding of the mechanisms underlying leg sensory bristle patterning, this process is not fully understood. Here, we consider two aspects of this process that have not been previously addressed. First, we investigate the regulation of the A/P-*hairy* stripes, which are of interest because they are expressed at a distance from the sources of morphogens that are known to pattern the leg disc. The early events of leg development are fairly well understood. The leg imaginal disc is divided into anterior and posterior compartments. Posterior compartment cells produce the Hedgehog (Hh) signal which is secreted and signals to anterior compartment cells to activate a stripe of high-level *dpp* expression dorsally and a stripe of *wingless* (*wg*) expression ventrally, leading to the formation of dorsal and ventral organizers (Diaz-Benjumea et al., 1994). Dpp, secreted by the dorsal organizer, specifies a dorsal leg fate and patterns the dorsal leg along the A/P axis in a concentration-dependent manner. Similarly, Wg specifies a ventral leg fate and organizes pattern along the A/P axis of the ventral leg (Brook and Cohen, 1996; Jiang and Struhl, 1996; Penton and Hoffmann, 1996; Struhl and Basler, 1993; Theisen et al., 1996). We have previously reported that the D/V-*hairy* stripes are established in direct response to the Hh, Dpp and Wg signals and that *hairy* acts as a critical link between *ac* and these morphogens (Hays et al., 1999; Kwon et al., 2004). Hh induces D/V-*hairy* expression near the A/P boundary, and Dpp and Wg act in concert with Hh to maintain D/V-*hairy* expression in its defined domain near the A/P boundary (Hays et al., 1999; Kwon et al., 2004). In this study, we demonstrate that distinct modes of regulation are utilized to establish the D/V-*hairy* and A/P-*hairy* longitudinal stripes. Rather than being locally induced, A/P-*hairy* expression is activated broadly along the leg circumference and Dpp and Wg function, respectively, to define the dorsal and ventral boundaries of A/P-*hairy* expression via repression.

A second aspect of leg microchaete patterning addressed in this study is the identity of factors that function in conjunction with Hairy to spatially define *ac* expression to the microchaete proneural fields. Since only four of the eight *ac* interstripe domains are established by Hairy, other as yet unidentified factors are likely to be involved in regulation of proneural *ac* expression. To distinguish between the four *ac* interstripes that express *hairy* and the four interstripes that do not express *hairy*, the *ac* interstripes will be referred to as either the *hairy*-ON or *hairy*-OFF interstripes. We identify the N ligand Dl (reviewed by Lai, 2004) as a second prepattern gene that together with *hairy* defines periodic *ac* expression. The potential function of Dl as a regulator of proneural *ac* expression was suggested by studies in the notum on which mechanosensory microchaetae are also organized in longitudinal rows (Parks et al., 1997). Hairy does not function in patterning the notal microchaetae (Simpson et al., 1999). Rather, it has been shown that Dl/N signaling sets up periodic *ac* expression in the notal microchaete primordia (Parks et al., 1997). We find that, as in the notum, *Dl* expression is up-regulated in stripes that overlap the *ac*-expressing microchaete proneural fields in the leg and that Dl signals to adjacent cells to repress *ac* expression. Surprisingly, however, unlike the notum, Dl function is only required within a subset of *ac* interstripes, the *hairy*-OFF interstripes. Consistent with this observation, N signaling, assayed by expression of two independent and widely expressed N-responsive reporters, is not activated (with one exception) in the *hairy*-ON stripes but rather in the complementary set of *hairy*-OFF interstripes. We further find that, like *ac*, periodic *Dl* expression in the leg is regulated by *hairy*. These observations suggest that Hairy and Dl function concertedly and nonredundantly to define *ac* expression within the leg microchaete proneural fields and that distinct mechanisms are utilized to generate the similar microchaete pattern on the leg and notum. Furthermore, observations from this and previous studies elucidate a general

pathway for establishment of periodic pattern in the leg and provide insight into the connections between morphogen function and generation of specific morphological features in the *Drosophila* adult leg.

Materials and methods

Fly strains and genetics

The following fly strains were used in this study: OregonR, *H15-lacZ* (Brook et al., 1993); *y w hsp70-flp; tkv^{a12} FRT40A/CyO* (Nellen et al., 1996), *dsh^{v6}FRT18A*/FM7, UAS-*arm*^{S10} (Pai et al., 1997), UAS-*tkv*^{Q253D} (Nellen et al., 1996), UAS-*N*^{intra} (Kidd et al., 1998), UAS-*Dl* (Jacobsen et al., 1998), UAS-*hairy* (Myat and Andrew, 2002) *cosh; h^{C1}/h^{5h07}TM6* [*cosh* is a 28 kb transgene which contains the *hairy* transcribed region and 14 kb of 5′ and 3 kb of 3′ sequences (Rushlow et al., 1989)], *N*^{55e11} *FRT18A/FM7* (Rulifson and Blair, 1995), *wπM5A πM10D FRT18A* and *wπM21C πM36F FRT40A* (Xu and Rubin, 1993), *Mad$^{1.2}$ FRT40A/CyO* (Kim et al., 1997), *w Ubi-GFP(S65T)nls FRT18A, Dl^{RF}/TM6B, Tb* and *ss Df*^{6B} *e/TM6B, Tb* (Parks et al., 1997), *w; Gbe+ Su(H)$_{m8}$-lacZ/TM3 Sb* (Furriols and Bray, 2001), *w; rn^{GAL4-5}/TM3, ftz/lacC, ry Sb Ser* (St Pierre et al., 2002), *sc$^{10-1}$/y ac*$^{Hw-1}$ (double mutant in the *ac* and *sc* genes with a deficiency breakpoint just 5′ to *ac* start of transcription and a nonsense mutation in *sc* gene; adult flies lack almost all sensory organs (Romani et al., 1989)).

Dl^{RF}/ss *Df*^{6B}*e* animals were generated by mating *Dl*^{RF}*/TM6B Tb* virgin females with *ss Df*^{6B}*/TM6B Tb* males at the permissive temperature of 18°C. Larvae were raised at the permissive temperature until 2 h APF. Then, the nontubby prepupae were incubated at a nonpermissive temperature of 32°C for 3 h and dissected after a 1 h recovery period at 25°C.

tkv, *Mad*, *dsh* and *N* mutant clones were made in larvae of the genotypes: *fhsp70-flp; tkv^{a12} FRT40A/π*M 36F FRT40A, *yhs-flp;Mad$^{1.2}$ FRT40A/πM 36F FRT40A, dsh^{v6}FRT18A/πM10D FRT18A*; *hs-flp*/+ or *N*^{55e11} *FRT18A/Ubi-GFP (S65T)nls FRT18A*; *hs-flp*/+. Clones were generated by heat shocking larvae (48–96 h AEL) for 1 h at 37°C. For Myc-marked clones, 3rd instar larvae or prepupae were heat-shocked prior to dissection for 1 h at 37°C to induce *πMyc* expression and were then allowed to recover at 25°C for 1 h prior to dissection.

For ectopic expression studies, leg imaginal discs were dissected from larvae or prepupae of the following genotypes: UAS-*tkv*^{Q253D}/+; *rn-Gal4*/+, UAS-*arm*^{S10}/+; *rn-Gal4*/+, UAS-*arm*^{S10}/+; *rn-Gal4*/+; UAS-*N*^{intra}/+; *rn-Gal4*/+, UAS-*Dl*/+; *rn-Gal4*/+, UAS-*hairy*/+; *rn-Gal4*/+.

Immunohistochemistry and microscopy

For all antibody stainings, pupal legs dissected between 4 and 6 h after puparium formation (APF) were treated as previously described (Carroll and Whyte, 1989). Primary antibodies used included: mouse anti-Myc, 1:5 (Xu and Rubin, 1993), rabbit–anti-β-gal, 1:2000 (R. Holmgren, unpublished), mouse anti-Hairy, 1:5 and rabbit anti-Hairy, 1:200 (Carroll et al., 1988), mouse anti-Achaete, 1:10 (Skeath and Carroll, 1991), rat anti-Ci, 1:1 (Motzny and Holmgren, 1995), rat anti-Serrate, 1:1000 (Papayannopoulos et al., 1998), mouse anti-CD2, 1:1000 (Serotech). Mouse anti-Delta, 1:400 (Qi et al., 1999) and mouse anti-N-extracellular domain, 1:25,000 (Diederich et al., 1994), were obtained from the Developmental Studies Hybridoma Bank.

All images were collected on a Zeiss Axiovert 200M equipped with ApoTome and a digital camera. Fluorescent images were collected as *Z*-stacks and subjected to 3D deconvolution or directly collected as apotomized *Z*-stacks.

Results

The A/P-hairy stripes are spatially defined by Dpp and Wg signaling

In the tarsus of T2 mid-prepupal legs, *ac* is expressed in eight and *hairy* in four longitudinal stripes along the leg circumference (Orenic et al., 1993). Expression of these stripes relative to the compartment boundary is shown diagrammatically in Fig. 1A. Analysis of legs with compromised *dpp* or *wg* function suggests that the division between the Dpp and Wg domains of influence runs along bristle row 7 in the anterior compartment and bristle row 2 in the posterior compartment (Fig. 1A) (Held and Heup, 1996). This would imply that the A/P-*hairy* stripes, which are positioned dorsal to *ac* stripes 7 and 2, respectively, are expressed in a dorso-lateral region of the leg. To more precisely determine the position of the A/P-*hairy* stripes, we compared *hairy* expression to that directed by *H15-lacZ*, an enhancer trap that marks ventral leg cells (Brook et al., 1993). Both the A and P-Hairy stripes are positioned just dorsal to the *H15-lacZ* expression domain consistent with the suggestion that they are expressed in the dorso-lateral region of the leg (Figs. 1B–C).

Since the A/P-*hairy* stripes appear to be expressed in cells located within a domain of Dpp influence, but at a distance from the Dpp source, we hypothesized that A/P-*hairy* expression might be activated by low levels of Dpp signaling. To test this hypothesis, *hairy* expression was assayed in clones lacking function of a Dpp receptor subunit, Thickveins (Tkv), or the transcriptional mediator of the Dpp pathway, Mothers against-dpp (Mad) (reviewed by Tabata, 2001). Somatic clones lacking *tkv* or *Mad* function were generated by FLP/FRT-mediated mitotic recombination (Xu and Rubin, 1993). In clones lacking either *tkv* (not shown) or Mad function (Figs. 2A–C), A/P-*hairy* expression is not compromised (Figs. 2A–C), suggesting that

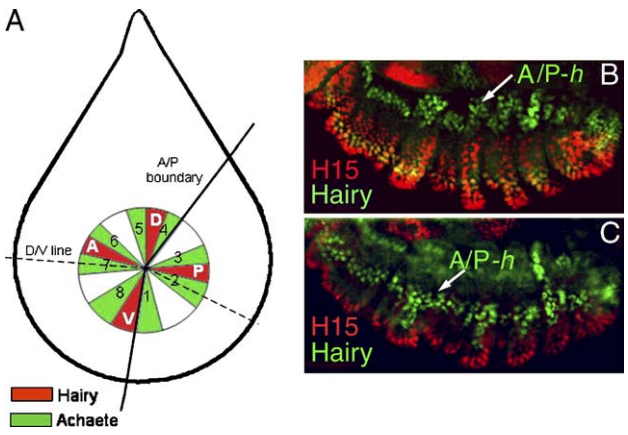

Fig. 1. The A/P-*hairy* stripes are expressed in the dorso-lateral region of prepupal legs. (A) Diagram summarizing the expression of *hairy* and *ac* in the tarsus of the 2nd leg at 6 h APF. This pattern is not observed until 6 h APF, but for the sake of clarity, the stripes are projected onto a diagram of a 3rd instar leg disc (dorsal is up and anterior is left). The circle represents the tarsus (individual tarsal segments are not depicted). *ac* (green) is expressed in eight longitudinal stripes, 3 in the posterior compartment and 5 in the anterior compartment. *hairy* (red) is expressed in four stripes between alternating pairs of *ac* stripes and defines periodic *ac* expression through repression. The D, V, A and P-*hairy* stripes are marked. D/V-*hairy* expression is activated during the 3rd larval instar, while the A/P-*hairy* stripes are not expressed until 3–4 h APF. (B–C) Comparison of Hairy (anti-Hairy, green in panels B–C, arrows indicate the A/P-*hairy* stripes) to *H15-lacZ* expression (anti-β-Gal, red in panels B–C), which marks ventral leg cells in a prepupal leg dissected between 4 and 5 h APF (dorsal is up and distal is right). Two focal planes of the same leg are shown to allow visualization of both A/P-*hairy* stripes. Both A and P-*hairy* stripes are positioned dorsal to the *H15-lacZ* domain of expression.

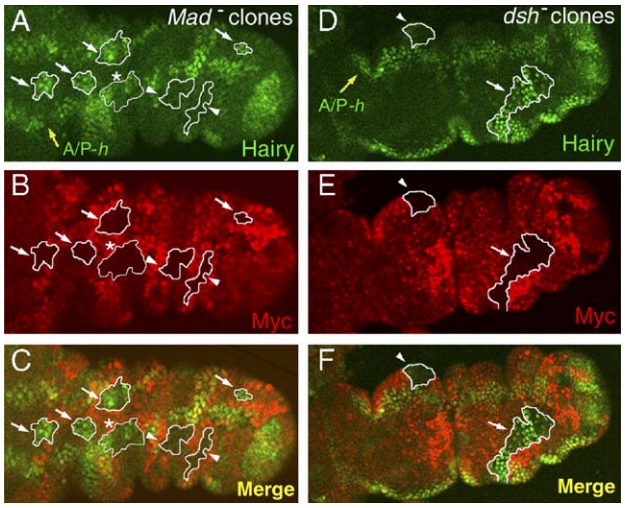

Fig. 2. A/P-*hairy* expression is spatially defined by Dpp and Wg signaling. (A–C) Expression of Hairy (anti-Hairy, green in panels A, C) in $Mad^{1.2}$ clones was examined in a prepupal leg dissected between 4 and 5 h APF. Clones (outlined in white) are marked by lack of Myc expression (anti-Myc, red in panels B, C). A/P-Hairy (yellow arrow in panel A) expression is not compromised in a clone which overlaps the stripe (asterisk). Clones positioned dorsal (arrows) to the A/P-Hairy stripe ectopically and cell-autonomously express Hairy, whereas ectopic Hairy expression is not observed in ventral clones (arrowhead). (D–F) Expression of Hairy (anti-Hairy, green in panels D, F) in dsh^{v26} clones was examined in a prepupal leg dissected between 4 and 5 h APF. Clones (outlined in white) are marked by lack of Myc expression (anti-Myc, red in panels E, F). A large clone positioned ventral (arrow) to the A/P-Hairy stripe (yellow arrow) exhibits ectopic Hairy expression, whereas ectopic Hairy expression is not observed in a dorsal clone (arrowhead).

Dpp function is not required to activate A/P-*hairy* expression. Instead, we observe that compromised Dpp signaling results in ectopic *hairy* expression specifically in the dorsal region of the leg (Figs. 2A–C). It is unlikely that inappropriate *hairy* expression in *Mad* and *tkv* clones is a result of ectopic activity of the D/V-*hairy* enhancers since we have previously shown that the D/V-*hairy* stripes are activated by Ci (Hays et al., 1999; Kwon et al., 2004), the transcriptional mediator of the Hh pathway (reviewed by Koebernick and Pieler, 2002; Ruiz i Altaba, 1999) and, therefore, can only be expressed near the A/P compartment boundary within cells that receive high levels of the Hh signal.

The finding that *hairy* is ectopically expressed in *tkv* and *Mad* mutant clones suggests that A/P-*hairy* expression is repressed by Dpp, and we would, therefore, expect that elevation of Dpp signaling within A/P-*hairy* expressing cells would abrogate A/P-*hairy* expression. Dpp signaling was elevated throughout most of the tarsus by expressing a constitutively active form of Tkv (UAS-tkv^{QD}) (Nellen et al., 1996) under control of *rotund*-Gal4 (*rn*-Gal4) (St Pierre et al., 2002), which directs *Gal4* expression from the distal half of the first tarsal segment through the proximal half of the fifth tarsal segment (Fig. 3F). Fig. 3E shows *hairy* expression in a wild-type prepupal leg dissected between 4 and 5 h after puparium formation (APF). At this stage, *hairy* is expressed in four longitudinal stripes, two A/P-*hairy* and two D/V-*hairy* stripes (two stripes are visible in Fig. 3E), and a series of transverse stripes, which partially encircle the tarsal segments. We observe that expression of tkv^{QD} under control of *rn-Gal4* results in disruption of A/P-*hairy* expression throughout most of the tarsus, while expression of the D/V-*hairy* stripes is largely unaffected (Figs. 3B, D). The A/P-Hairy and D/V-Hairy stripes were unambiguously distinguished by comparing *hairy* to *ci* expression. The D/V-*hairy* stripes are expressed along the compartment boundary, which is marked in Figs. 3A–D by the border of *ci* expression. Some residual A/P-*hairy* expression is apparent, which is likely due to heterogeneous expression directed by the *rn-Gal4* driver (Fig. 3F). Taken together, these results suggest that Dpp acts to define the dorsal boundary of A/P-*hairy* expression.

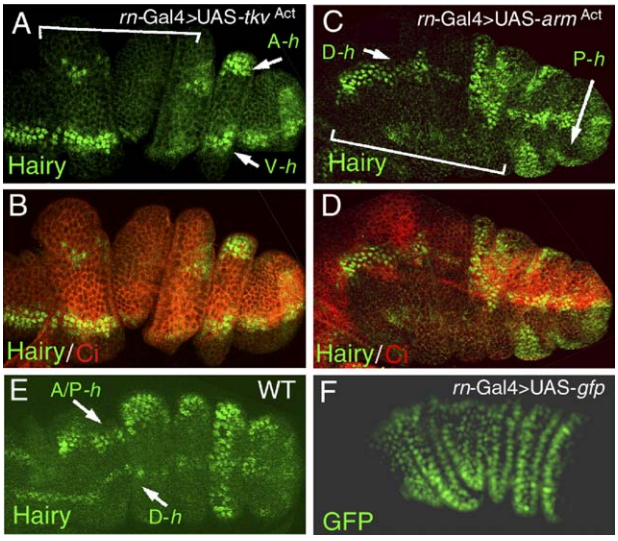

Fig. 3. Ectopic Dpp or Wg signaling disrupts A/P-*hairy* expression. (A–B) Expression of *hairy* (anti-Hairy, green in panels A, B) in a prepupal leg dissected between 4 and 5 h APF, expressing a constitutively active form of the Dpp receptor Tkv (UAS-tkv^{QD}) under control of *rn-Gal4* [which directs *Gal4* expression from the distal half of the first tarsal segment through the proximal half of the fifth tarsal segment (see panel F)]. Ci expression (anti-Ci, red in panel B) marks the A/P compartment boundary. A-Hairy (A-*h*, arrow) and V-Hairy (V-*h*, arrow) stripes are seen in the focal plane shown. The A- and V-Hairy stripes can be distinguished by virtue of the coincidence of the compartment boundary and V-*hairy* expression. While A-*hairy* expression in the distal leg is unaffected (arrow), A-*hairy* expression is drastically reduced throughout much of the proximal tarsus of this leg (region with reduced expression is indicated by a bracket). V-*hairy* expression is largely unaffected. Residual A/P-*hairy* expression is likely due to heterogeneous expression directed by the *rn-Gal4* driver. (C–D) Expression of Hairy (anti-Hairy, green in panels C, D) in a prepupal leg, dissected between 4 and 5 h APF, expressing a constitutively active form of Arm (UAS-arm^{S10}) under control of *rn-Gal4*. Ci expression (anti-Ci, red in panel D) marks the A/P compartment boundary. P-Hairy (P-*h*, arrow) and D-Hairy (D-*h*, arrow) stripes are seen in the focal plane shown. The P and D-Hairy stripes can be distinguished by virtue of the coincidence of the compartment boundary with D-*hairy* expression. P-*hairy* expression is drastically reduced throughout the proximal tarsus of this leg (region with reduced expression is indicated by a bracket), while P-*hairy* expression in the distal leg is unaffected (arrow). D-*hairy* expression is also largely unaffected. (E) Expression of *hairy* (anti-Hairy, green in panels A, B) in a wild-type prepupal leg, dissected between 4 and 5 h APF. D-Hairy (D-*h*, arrow) and A/P-Hairy (A/P-h, arrow) stripes can be seen in this focal plane. Note that both these stripes extend along the entire length of the P/D axis. One transverse stripe, in the fourth tarsal segment, is also visible. (F) Expression of GFP (green) in a wild-type prepupal leg, dissected between 4 and 5 h APF, under the control of *rn-Gal4*.

The effects of Dpp signaling on A/P-*hairy* expression suggest that spatial regulation of *hairy* expression in the dorso-lateral leg may involve broad activation along the leg circumference combined with repression to define the dorsal and ventral boundaries. Wg signal produced by the ventral organizer is an obvious candidate for a factor which might act to delimit A/P-*hairy* expression ventrally. We asked whether Wg plays a role in regulation of A/P-*hairy* expression by examining *hairy* expression in clones lacking function of a transducer of the Wg signal, Dishevelled (Dsh) (reviewed by Seto and Bellen, 2004). In clones lacking *dsh* function, ectopic *hairy* expression is observed in ventral but not dorsal clones (Figs. 2D–F). Consistent with this finding, we observe that *hairy* expression is abrogated when the level of Wg signaling is raised in the dorso-lateral leg by expression of a constitutively active form of a transcriptional transducer of Wg signaling, Armadillo (UAS-arm^{S10}) (Pai et al., 1997), under control of *rn-Gal4* (Figs. 3C–D). These observations support the hypothesis that Wg acts to define the ventral boundary of the stripes of A/P-*hairy* expression.

Delta is expressed in the leg microchaete proneural fields

Since *hairy* function is not sufficient to regulate periodic *ac* expression in prepupal legs, we sought to identify other factors that might regulate *ac*. It has previously been reported that *Dl* expression is uniformly distributed throughout leg discs but is elevated in series of rings in the proximal region of each segment (Bishop et al., 1999; de Celis et al., 1998; Rauskolb and Irvine, 1999). We examined *Dl* expression at various times APF and found that, beginning at approximately 4 h APF, *Dl* expression is up-regulated in two longitudinal stripes (Figs. 4A, B) and that, by 6 h APF, there are eight Dl stripes, one on either side of each of the four Hairy stripes (Figs. 4C, D). Expression of *Dl* adjacent to the *hairy* stripes is reminiscent of *ac* expression at 6 h APF and indicates that *Dl* expression is elevated within the microchaete proneural fields. A second N ligand, Serrate (Ser), is expressed in rings along the leg's proximal/distal (P/D) axis and is required for leg segmentation, as is Dl (Bishop et al., 1999; de Celis et al., 1998; Rauskolb and Irvine, 1999). An antibody against Ser (Papayannopoulos et al., 1998) was used to visualize its expression in prepupal legs at 6 h APF, but we do not find that its expression is elevated in the microchaete proneural fields (not shown), suggesting that Ser is unlikely to function in regulation of proneural *ac* expression in the leg. In the notum, N is expressed in a pattern complementary to *Dl* expression. We examined the distribution of the N receptor by utilizing an antibody against its extracellular domain (Diederich et al., 1994), but although N levels are higher in rings along the P/D axis, we do not observe obvious elevated expression in longitudinal stripes (Fig. 4E).

Delta establishes periodic achaete expression

The spatial and temporal pattern of *Dl* expression in prepupal legs suggested that Dl/N signaling might function to regulate proneural *ac* expression. To investigate the role of Dl/N signaling in regulating proneural *ac* expression, *ac* expression was assayed in clones lacking N function. As shown in Figs. 5A–A″, we observe that loss of N function results in ectopic expression of *ac* in clones within some but not all Ac interstripes. A potential explanation for the lack of ectopic expression in some clones is that N signaling is not required for repression of *ac* in the *hairy*-ON interstripes. To determine if this is the case, we examined *ac* expression in prepupal legs of the genotype: Dl^{RF}/Dl^{6B} (both are temperature sensitive alleles). Dl^{RF}/Dl^{6B} white prepupae were incubated at the nonpermissive temperature from 2 to 5 h APF and dissected after a 1 h recovery period (see Materials and methods for details). We find that, in prepupal legs with compromised *Dl* function, *ac* expression expands but only into the *hairy*-OFF *ac* interstripes (Figs. 5B–B′; expression of *ac* in a wild-type prepupal leg is shown in Fig. 5C, for comparison). Furthermore, induction of widespread N signaling throughout the tarsus severely reduces proneural *ac* expression. Fig. 5D shows a leg expressing a constitutively active form of N [*UAS-N-intra* (*Ni*)] (Kidd et al., 1998) under control of *rn-Gal4*. In this leg, most of the tarsus is overgrown, and there is little *ac* expression

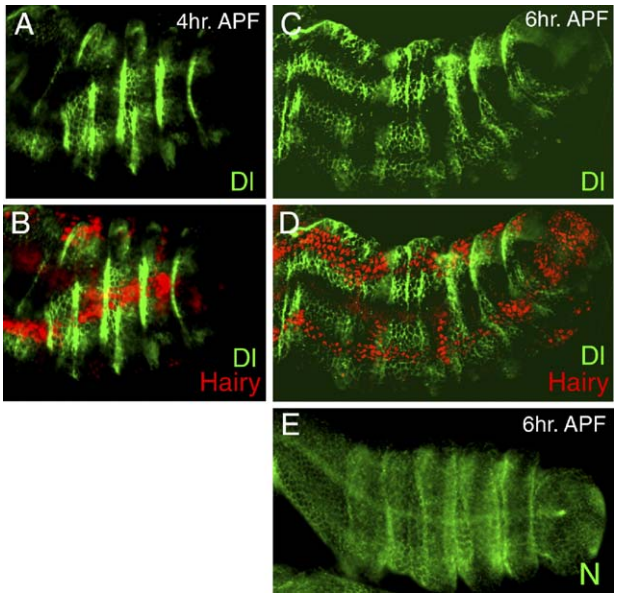

Fig. 4. Delta expression is elevated in the microchaete proneural fields. (A–B) Comparison of *Dl* (anti-Dl, green in panels A, B) and *hairy* expression (anti-Hairy, red in panel B) in a prepupal leg dissected at approximately 4 h APF. *Dl* expression is up-regulated in two longitudinal domains at this stage (the rings along the P/D axis are also visible). At this stage, the stripes appear fused in some regions along the P/D axis. In later stages, *Dl* expression is further down-regulated between the stripes. *hairy* is expressed between the pair of Dl stripes indicating that *Dl* expression overlaps cells which will comprise the *ac* microchaete proneural fields. (C–D) Comparison of Dl (anti-Dl, green in panels C, D) to *hairy* expression (anti-Hairy, red in panel D) in a prepupal leg dissected at 6 h APF. *Dl* expression is up-regulated in eight longitudinal (four are visible in this focal plane) domains at this stage (some of the rings along the P/D axis are still visible). A pair of Dl stripes is expressed on either side of each Hairy stripe at this stage, reminiscent of *ac* expression at this stage. (E) Expression of N (anti-N, green) in a wild-type prepupal leg dissected at approximately 6 h APF. Although elevated N expression in rings along the P/D axis is evident, we do not observe elevated expression in longitudinal domains. The stripe of N staining in the center of the leg corresponds to an internal structure.

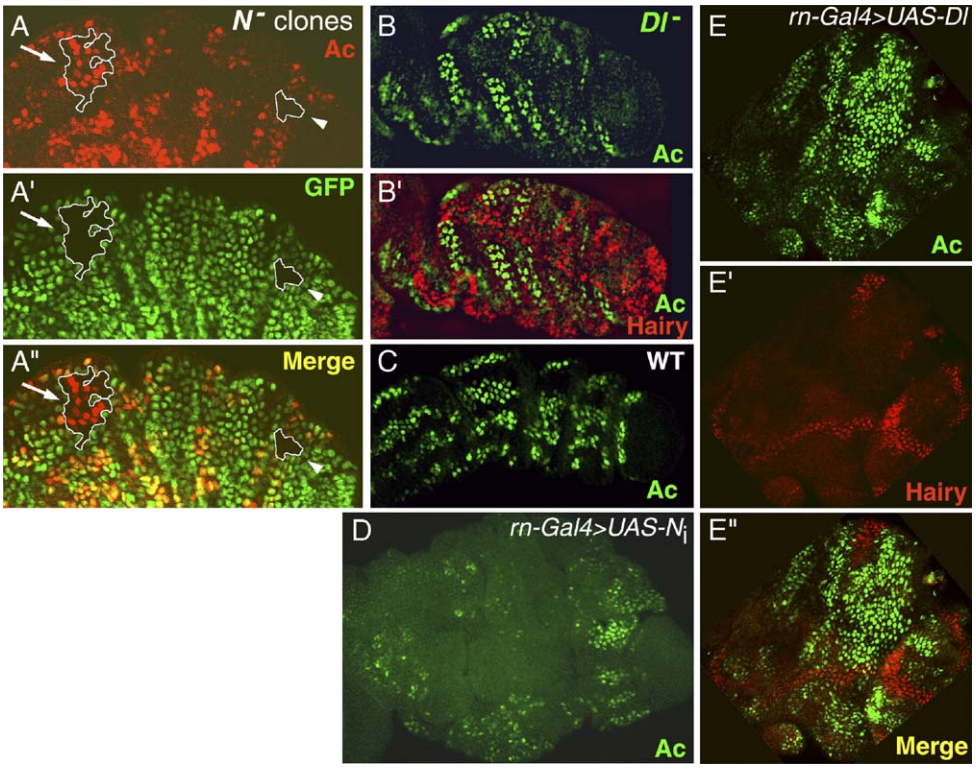

Fig. 5. Delta establishes periodic *ac* expression. (A–A′′) Expression of *ac* (anti-Ac, red in panels A, A′′) in N^{55e11} clones was examined in a prepupal leg dissected at 6 h APF. Clones (outlined in white) are marked by lack of GFP expression (green in panels A′, A′′). Arrow indicates a clone between two Ac stripes, in which there is ectopic *ac* expression. Another clone (arrowhead) in the adjacent *ac* interstripe, which presumably corresponds to a *hairy* expression domain, does not exhibit ectopic Ac expression. (B–B′) Expression of Ac (anti-Ac, green in panels B–B′) and Hairy (anti-Hairy, red in panel B′) in prepupal legs, dissected at 6 h APF, of the genotype Dl^{RF}/Dl^{6B}. *ac* expression expands into the *ac* interstripe domains which do not express *hairy* (*hairy*-OFF interstripes), suggesting that Dl functions in the set of *ac* interstripe domains, complementary to the *hairy*-expressing interstripes, to repress proneural *ac* expression. (C) Expression of *ac* (anti-Ac, green) in a wild-type prepupal leg dissected at 6 h APF. (D) Expression of *ac* (anti-Ac, green) in a prepupal leg, dissected at 6 h APF, expressing a constitutively active form of N (UAS-N^{intra}) under control of *rn-Gal4*. Note the overgrowth of the tarsus and that there is very little *ac* expression throughout this region, while expression of *ac* in longitudinal stripes persists in the distal tarsus. (E–E′′) Expression of *ac* (anti-Ac, green in panels E, E′′) and *hairy* (anti-Hairy, red in panels E′, E′′) in a prepupal leg dissected at 6 h APF, expressing *Dl* (UAS-*Dl*) under control of *rn-Gal4*. *ac* expression is elevated and almost uniform but is excluded from *hairy*-expressing cells.

throughout this region, but expression of *ac* in longitudinal stripes persists in the distal tarsus. Combined, these findings reveal a role for Dl/N signaling in regulation of *ac* expression in the microchaete proneural fields and suggest that Dl, made in cells that comprise the proneural fields, signals to adjacent cells to activate N signaling and repress *ac* expression.

N signaling is activated in the hairy-OFF ac interstripes

Genetic studies suggest that *hairy* and *Dl* function nonredundantly to repress *ac* expression in complementary interstripe domains. This would imply that N signaling is activated in the *hairy*-OFF interstripes but not in the *hairy*-ON interstripes or within the proneural fields. In order to visualize N signaling and determine where it is activated in prepupal legs, we utilized two N-responsive reporters, *Gbe+Su(H)m8-lacZ* (Furriols and Bray, 2001) and *E(spl)mβ-CD2* (de Celis et al., 1998). *Gbe+Su(H)m8-lacZ* has been shown to accurately reflect N signaling in the wing (Furriols and Bray, 2001). *E(spl)mβ* is a member of the *Enhancer of split* Complex [E-(spl)-C] of genes (Delidakis and Artavanis-Tsakonas, 1992; Klambt et al., 1989; Knust et al., 1992), which are well-characterized targets of N (Bailey and Posakony, 1995; Furukawa et al., 1995; Lecourtois and Schweisguth, 1995). *E(spl)mβ* is the most widely expressed N-target gene (Cooper et al., 2000; de Celis et al., 1996; Nellesen et al., 1999), and, in discs, the *E(spl)mβ-CD2* reporter is expressed in a pattern similar to *Gbe+Su(H)m8-lacZ* expression (Furriols and Bray, 2001). We observe that these two independent reporters direct similar expression in prepupal legs (Figs. 6A–D′). *Gbe+Su(H)m8-lacZ* and *E(spl)mβ-CD2* are each expressed at the distal edge of each leg segment, regions in which N signaling is known to be activated in prepupal legs (Bishop et al., 1999; de Celis et al., 1998; Rauskolb and Irvine, 1999). In addition to the rings of expression along the proximal/distal axis, we consistently observe five longitudinal stripes of *Gbe+Su(H)m8-lacZ* and *E(spl)mβ-CD2* expression in the basitarsus of T2 prepupal legs. Four stripes are expressed in *hairy*-OFF interstripes (Figs. 6A–D′), consistent with genetic studies indicating that Dl/N signaling is required within the *hairy*-OFF interstripe domains.

The fifth *Gbe+Su(H)m8-lacZ* and *E(Spl)mβ-CD2* stripes overlap one of the four Hairy stripes (Figs. 6A–A′, C–C′), which was identified as the V-Hairy stripe by comparing *Gbe+Su(H)m8-lacZ* to *wg* expression (Figs. 6H–H′). *wg* is expressed in the ventral leg (Baker, 1988) and overlaps the V-Hairy stripe (Hays et al., 1999), and we observe that one *Gbe+Su*

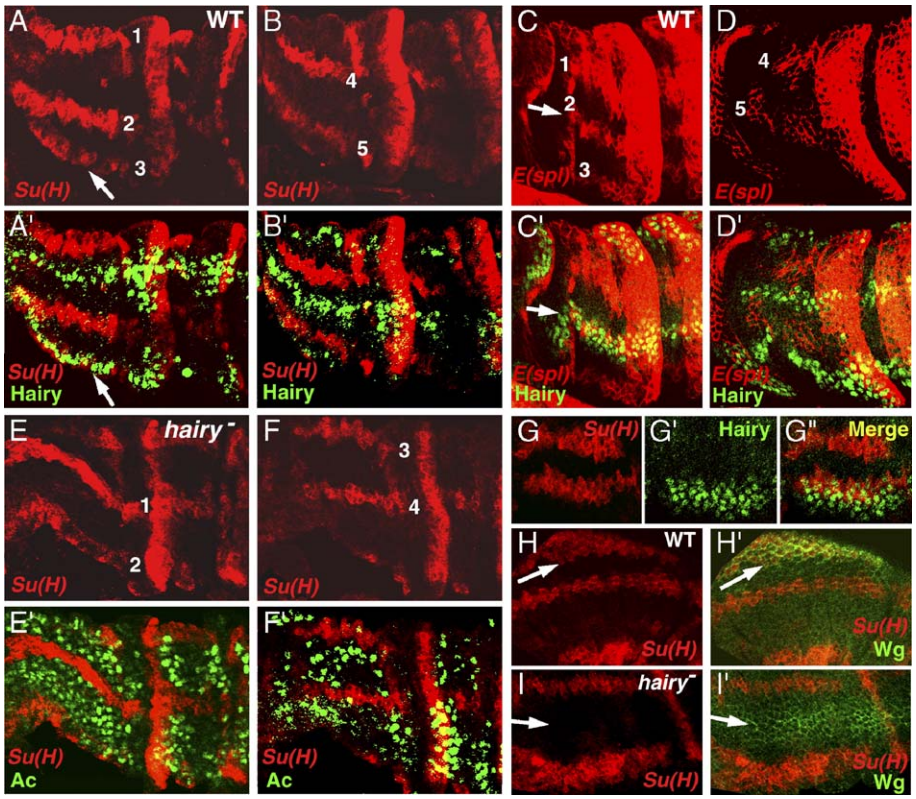

Fig. 6. N signaling is activated in striped domains complementary to the *hairy* expression domains. (A–B′) Comparison of *hairy* (anti-Hairy, green in panels A′, B′) and *Gbe+Su(H)m8-lacZ* expression (anti-β-Gal, red in panels A–B′) in a wild-type T2 prepupal leg dissected at 6 h APF. Two focal planes of the same leg are shown to allow visualization of all the *Gbe+Su(H)m8-lacZ* stripes. Five stripes are visible (numbered in panels A and B). Four *Gbe+Su(H)m8-lacZ* stripes are expressed in domains complementary to Hairy expression (A′ and B′), and one *Gbe+Su(H)m8-lacZ* stripe overlaps Hairy expression in the ventral leg (arrow in panels A and A′). (C–D′) Comparison of *hairy* (anti-Hairy, green in panels C′, D′) and *E(spl)mβ-CD2* expression (anti-CD2, red in panels C–D′) in a wild-type T2 prepupal leg dissected at 6 h APF. Two focal planes of the same leg are shown to allow visualization of all the *E(spl)mβ-CD2* stripes. Five stripes are visible (numbered in panels C and D). Four *E(spl)mβ-CD2* stripes are expressed in domains complementary to Hairy expression (C′ and D′), and one *E(spl)mβ-CD2* stripe overlaps Hairy expression in the ventral leg (arrow). (E–F′) Comparison of *ac* (anti-Ac, green in panels E′, F′) and *Gbe+Su(H)m8-lacZ* expression (anti-β-Gal, red in panels E–F′) in a T2 prepupal leg of the genotype: *cosh;* h^{C1}/h^{5h07}*TM6* (which lacks expression of *hairy* in the leg longitudinal stripes), dissected at 6 h APF. Two focal planes of the same leg are shown to allow visualization of all the *Gbe+Su(H)m8-lacZ* stripes. Note that only four *Gbe+Su(H)m8-lacZ* stripes are expressed, suggesting that *hairy* function is required for activation of N signaling in the 5th stripe. *ac* expression expands to fill most of the tarsus, except within cells that express *Gbe+Su(H)m8-lacZ*. (G–G″) Close-up comparison of *Gbe+Su(H)m8-lacZ* (anti-β-Gal, red in panels G, G″) and V-Hairy expression (anti-Hairy, green in panels G′, G″) in a wild-type T2 prepupal leg dissected at 6 h APF. V-Hairy and *Gbe+Su(H)m8-lacZ* partially overlap. The *Gbe+Su(H)m8-lacZ* extends one cell beyond the Hairy stripe, and the Hairy stripe extends about two cells beyond the *Gbe+Su(H)m8-lacZ* stripe. (H–H′) Comparison of *Gbe+Su(H)m8-lacZ* (anti-β-Gal, red in panels H, H′) and *wg* expression (anti-Wg, green in panel H′) in a wild-type T2 prepupal leg. One *Gbe+Su(H)m8-lacZ* overlaps *wg* expression in the ventral leg (arrow). (I, I′) Comparison of *Gbe+Su(H)m8-lacZ* (anti-β-Gal, red in panels I, I′) and *wg* expression (anti-Wg, green in panel I′) in a T2 prepupal leg of the genotype: *cosh;* h^{C1}/h^{5h07}*TM6*. Note that the *Gbe+Su(H)m8-lacZ* stripe that overlaps *wg* in the wild-type leg (H–H′) expression in absent (arrow).

(H)m8-lacZ stripe consistently overlaps *wg* expression (Figs. 6H–H′). This stripe will be designated as the V-*Gbe+Su(H)m8-lacZ* stripe. The overlap of V-*Gbe+Su(H)m8-lacZ* and *E(Spl)mβ*-CD2 expression with the V-Hairy stripe was surprising because it would suggest redundancy between *hairy* and *Dl/N* function in this region, and we have previously observed an absolute requirement for *hairy* function in the ventral leg. In the absence of *hairy* function, *ac* expression expands to fill all *hairy*-ON interstripes, and, in adult legs lacking *hairy* function, ectopic microchaete bristles are observed in the ventral leg (Orenic et al., 1993). A potential explanation for the apparent lack of redundancy between *hairy* and *Dl/N* function in the ventral leg is provided by a further observation. In the absence of *hairy* function, Dl/N signaling is compromised within the V-*hairy* expression domain. *Gbe+Su(H)m8-lacZ* and *ac* expressions were assayed in pupal legs of the genotype *cosh;* h^{C1}*/TM6 Ubx* h^{5h07}. *cosh* is a *hairy* transgene which rescues embryonic *hairy* function but does not express *hairy* in the longitudinal leg stripes (Rushlow et al., 1989). We find that, in legs that lack *hairy* function, there are, consistently, four rather than five stripes of *Gbe+Su(H)m8-lacZ* expression (Figs. 6E–F′) and that *ac* expression expands into the domains between the remaining *Gbe+Su(H)m8-lacZ* stripes. Comparison of *Gbe+Su(H)m8-lacZ* to *wg* expression (Figs. 6I–I′) shows that the V-*Gbe+Su(H)m8-lacZ* stripe is absent, while expression of other *Gbe+Su(H)m8-lacZ* stripes is unaffected (Figs. 6E–F′, I–I′). Hence, the concomitant loss of *hairy* function and N signaling in *cosh;* h^{C1}*/TM6 Ubx* h^{5h07} legs explains the phenotypes observed in the ventral leg when *hairy* function is compromised. Taken together, these results indicate that *hairy* and *Dl* function in alternate interstripe domains to define the periodicity of *ac* expression. An exception to this conclusion is that, in the ventral leg, where N

signaling and *hairy* expression overlap, both Hairy and Dl/N may contribute to *ac* repression. Careful comparison of *hairy* and *Gbe+Su(H)m8-lacZ* expression shows that they do not completely overlap (Figs. 6G–G′′): *hairy* expression extends 2 cells posterior to the *Gbe+Su(H)m8-lacZ*, and *Gbe+Su(H)m8-lacZ* extends about one cell anterior to the Hairy stripe. Hence, Hairy and Dl/N may function together to repress *ac* expression in a broader domain than either would alone.

Regulation of Delta expression

In the notum, *Dl* expression in the microchaete proneural fields is initially independent of *ac* function, but *ac* is required at later stages to maintain *Dl* expression (Parks et al., 1997). We find that, in the leg, *ac/sc* function is not required for expression of *Dl* before 7 h APF. *Dl* expression was examined in legs dissected between 3 and 7 h APF from $sc^{10\text{-}1}$/Y males, which lack *ac* and *sc* function (Romani et al., 1989), and we observed that *Dl* expression is not compromised at any stage between 3 and 7 h (Fig. 7A). We were not able to look beyond 7 h due to formation of cuticle, which impedes antibody staining. The complementary expression of *Dl* and *hairy* suggests *hairy* as a potential regulator of *Dl*. To assess a potential function for *hairy* in regulation of *Dl* expression, *Dl* expression was assayed in *cosh;* h^{C1}*/TM6 Ubx* h^{5h07} legs. As mentioned, in legs of this genotype, *ac* expression expands into the *hairy*-ON interstripes, and we find that *Dl* expression expands into these domains as well (Fig. 7B), resulting in four broad longitudinal domains of elevated *Dl* expression. Consistently, we find that ectopic expression of *hairy* results in loss of *Dl* expression (Figs. 7C–C′′). These observations suggest that the longitudinal stripes of *Dl* expression are established in part through repression by Hairy. Since, in the notum, *ac* function is required to maintain *Dl* expression, it is possible that the expansion of *Dl* expression is due to ectopic *ac* expression in *hairy* mutants. However, this is unlikely as we observe the same effect on *Dl* expression in *hairy* mutant prepupal legs younger than 6 h APF, before *ac* expression in the microchaete proneural fields is activated (Fig. 7A).

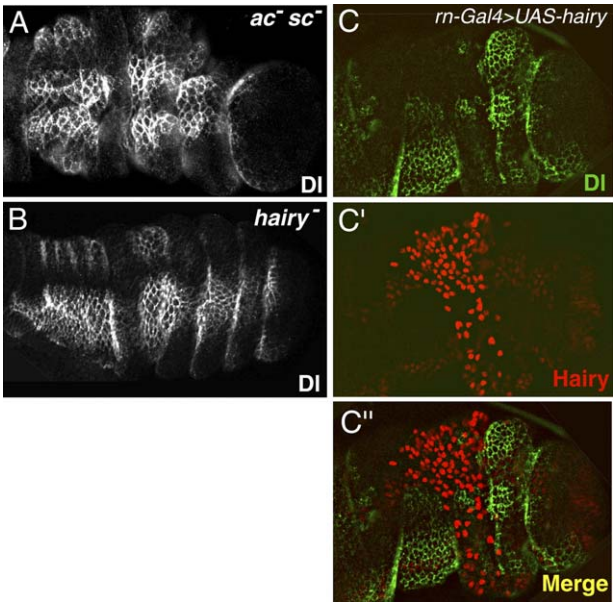

Fig. 7. Hairy establishes periodic Delta expression. (A) Expression of *Dl* (anti-Dl) in a prepupal leg of the genotype, $sc^{10\text{-}1}$/Y (which lacks both *ac* and *sc* function), dissected between 4 and 5 h APF. *Dl* expression in longitudinal stripes is not affected by loss of *ac* and *sc* function. (B) Expression of *Dl* (anti-Dl) in a prepupal leg of the genotype *cosh;* h^{C1}/h^{5h07}*TM6*, dissected at approximately 6 h APF. In the absence of *hairy* function, *Dl* expression expands into four broad domains. (C–C′) Comparison of *Dl* (anti-Dl, green in panels C, C′′) and *hairy* expression (anti-Hairy, red in panels C′, C′′) in a prepupal leg, dissected between 4 and 5 h APF, expressing *hairy*, (UAS-*hairy*) under control of *rn-Gal4*. Note that *Dl* expression is lost or very reduced in *hairy*-expressing cells.

The expansion of *Dl* expression in legs lacking *hairy* function might explain the loss of the V-*Gbe+Su(H)m8-lacZ* stripes in *hairy* mutant legs. Previous studies have shown that cells expressing high levels of *Dl* or *Ser* exhibit a "dominant-negative" effect, that is, they do not activate N signaling (Micchelli et al., 1997). Hence, elevated *Dl* expression in the V-*hairy* domain of expression could result in loss of N signaling in this region. To determine whether high-level *Dl* expression interferes with N signaling in the leg *ac* interstripe domains, *ac* expression was examined in prepupal legs expressing *UAS-Dl* (Jacobsen et al., 1998) under control of *rn-Gal4*. This results in elevated and almost uniform *ac* expression along the leg circumference, except that *ac* expression is excluded from *hairy*-expressing cells (Figs. 5E–E′′).

Discussion

Distinct modes of hairy regulation along the A/P and D/V axes

Patterning of the leg imaginal disc along its circumference axis is controlled by the Hh, Dpp and Wg morphogens. In this and previous studies, we have sought to elucidate the molecular mechanisms through which these signals give rise to specific morphological features of the leg, the mechanosensory microchaetae. We have shown that patterning of leg mechanosensory microchaetae requires spatially defined expression of the proneural gene *ac* and its repressor Hairy. Expression of *hairy* in two pairs of longitudinal stripes, the D/V-*hairy* and A/P-*hairy* stripes, is directed by separate enhancers that are Hh-, Dpp- and Wg-responsive. In this study, we report that the D/V-*hairy* and A/P-*hairy* stripes are differentially regulated by Dpp and Wg and that distinct mechanisms are utilized to control *hairy* expression along the A/P and D/V axes. D/V-*hairy* expression is locally induced near the A/P compartment boundary by Hh signaling. In addition, Dpp and Wg positively influence expression of the dorsal and ventral components of the D/V-*hairy* stripes, respectively, by acting together with Hh to define the register of these stripes relative to the compartment boundary (Hays et al., 1999; Kwon et al., 2004). On the other hand, the A/P-*hairy* stripes, which are expressed orthogonal to the D/V-*hairy* stripes and A/P compartment boundary, are not activated via local induction. Rather, it appears that they are broadly activated along the leg circumference and repressed by Dpp dorsally and Wg ventrally to define their dorsal and ventral boundaries. This model for A/P-*hairy* regulation is supported by the observations that *hairy* is ectopically expressed in dorsal,

but not ventral, clones lacking *tkv* or *Mad* function and that A/P-*hairy* expression is compromised by elevation of Dpp signaling. Furthermore, ventral, but not dorsal, clones lacking *dsh* function also ectopically express *hairy* and high-level Wg signaling results in loss of A/P-*hairy* expression.

A potential caveat to our model for regulation of A/P-*hairy* expression is that conclusions were drawn from analysis of endogenous *hairy* expression rather than by examining expression directed by isolated A/P-*hairy* enhancer(s). Hence, it is possible that the ectopic *hairy* expression seen in *tkv*, *Mad* and *dsh* mutant clones is a result of expansion of D/V-*hairy* rather than A/P-*hairy* expression. However, several lines of evidence argue against this interpretation. First, through genetic and molecular analyses of D/V-*hairy* enhancer function, we have demonstrated that Dpp and Wg positively regulate D/V-*hairy* expression, an observation that is inconsistent with the suggestion that D/V-*hairy* is ectopically expressed in clones unable to respond to Dpp or Wg signaling. Furthermore, in 3rd instar and early prepupal leg discs, stages at which the A/P-*hairy* stripes are not expressed, ectopic *hairy* expression is not observed in *tkv* mutant clones (Bulanin and Orenic, unpublished results). Second, we have found that the D/V-*hairy* stripes can only be expressed in anterior compartment cells near the A/P boundary, which are the cells that receive and respond to Hh signal. Thus, it is unlikely that ectopic *hairy* expression observed in clones at distance from the compartment boundary, which receive little or no Hh signal, and in the posterior compartment, in which cells do not respond to Hh signal, corresponds to D/V-*hairy* expression. Finally, we find that elevation of Dpp or Wg signaling specifically disrupts A/P-*hairy* but not D/V-*hairy* expression. Taken together, these findings are consistent with the conclusion that A/P-*hairy* rather than D/V-*hairy* is expressed in clones compromised in their response to Dpp and Wg signaling.

The expression of the A/P-*hairy* stripes at a distance from the dorsal and ventral organizers implies that A/P-*hairy* expression is repressed even at low threshold levels of Dpp and Wg signaling. This raises questions regarding the mechanisms through which Dpp and Wg define the sharp boundaries of the A/P-*hairy* stripes. A mechanism for Dpp-mediated repression in imaginal discs has been described, in which a complex of activated Mad with the Schnurri transcription factor acts directly through a repression element in the *brinker* (*brk*) gene (Muller et al., 2003). However, Dpp does not establish sharp boundaries of *brk* expression. Rather, *brk* expression drops off in a graded fashion toward the source of Dpp. Since the dorsal boundary of A/P-*hairy* expression is sharp and at distance from the Dpp source, this would imply that A/P-*hairy* expression is very sensitive to Dpp-mediated repression. Hence, it will be of interest to further investigate this process. Also of interest are the mechanisms of Wg-mediated repression, which are poorly understood.

Prepattern function of Hairy and Delta

In this study, we identify *Dl* as a second prepattern gene that functions together with *hairy* to establish *ac* expression in the leg microchaete proneural fields. We present several lines of evidence that support this conclusion. First, we find that, beginning at 4 h APF, *Dl* expression is up-regulated in domains overlapping the microchaete proneural fields. This distribution of Dl is similar to that, in the notum, where Dl has been shown to regulate proneural *ac* expression (Parks et al., 1997). Second, we show that *ac* expression is expanded in legs with reduced *Dl* function. Third, we find that elevated N signaling throughout the tarsus results in severely reduced *ac* expression. Finally, we observe activation of N signaling within the *hairy*-OFF interstripes, in agreement with the genetic requirement for Dl/N signaling in these domains. Based on these results, we propose that *ac* expression is activated broadly during mid-prepupal leg development but is confined to the microchaete proneural fields by a previously generated prepattern of repression, established by Hairy and Dl/N signaling. This hypothesis is supported by analysis of *cis*-regulatory elements that direct *ac* expression in the leg microchaete proneural fields (Joshi and Orenic, unpublished). By generating rescue and reporter constructs, we have identified an enhancer that specifically controls expression of *ac* in the microchaete proneural fields. Unlike the *hairy* leg enhancers, we do not observe a modular organization of the *cis*-regulatory elements that control expression of *ac* stripes in different regions of the leg. Rather, preliminary analyses suggest that there is one enhancer consisting of an activation element that directs broad expression of *ac* along the leg circumference and two repression elements, which are N- or Hairy-responsive. This finding is consistent with genetic studies and our model for regulation of *ac* expression in the leg microchaete proneural fields.

hairy and *Dl* function to repress *ac* expression in complementary domains. *hairy* encodes a transcriptional repressor which has been previously shown to directly repress *ac* expression in the wing by binding a specific site in the *ac* promoter (Ohsako et al., 1994; Van Doren et al., 1994). It is likely that Hairy acts through a similar site to repress *ac* expression in the leg. Dl represses *ac* expression via a different mechanism: presumably, cells of the microchaete proneural fields, which express high levels of *Dl*, signal to adjacent cells to activate N. This suggestion is supported by the observation that expression of two N-responsive reporters is specifically activated in cells corresponding to the *hairy*-OFF interstripes. One of the reporters used in this study, *E(spl)mβ-CD2*, and other similar reporters recapitulate endogenous *E(spl)mβ-CD2* expression in wing and leg imaginal discs (de Celis et al., 1998; Furriols and Bray, 2001; Nellesen et al., 1999; Sotillos and De Celis, 2005). *E(spl)mβ* is one of seven genes in the E(spl)-C that encode bHLH repressors related to Hairy (Delidakis and Artavanis-Tsakonas, 1992; Klambt et al., 1989; Knust et al., 1992). Hence, it appears that *ac* expression in the leg microchaete proneural fields may be established by a prepattern of periodically expressed bHLH repressors.

N signaling is not activated within *ac*-expressing cells, even though these cells express high levels of *Dl*. This could be explained by a dominant-negative effect of Notch ligands on N signaling, which has been previously observed in the wing. In the wing, it has been shown that N signaling is not activated

within cells expressing high levels of Dl and Ser but, rather, that these cells signal to adjacent cells to activate N signaling within the wing margin (Micchelli et al., 1997). Consistent with the hypothesis of a potential dominant-negative function for Dl in the leg microchaete proneural fields is the observation that over-expression of *Dl* along the leg circumference results in expansion of *ac* expression into the *hairy*-OFF interstripes, which would be expected if N signaling was disabled. Over-expression of N ligand expression has been shown to exert a similar effect in other tissues (Doherty et al., 1996; Jacobsen et al., 1998; Klein et al., 1997; Micchelli et al., 1997).

A curious observation of this study is that, as suggested by genetic evidence and the expression of two N-responsive reporters, N signaling, with one exception, is not activated within the *hairy*-ON interstripes, even though each Hairy stripe is straddled on either side by a Dl stripe. This suggests either that Dl signals asymmetrically or that there is an asymmetric response to N signaling and raises questions regarding the underlying mechanism of asymmetric activation of N-target gene expression. A potential mechanism for asymmetric signaling by Dl is suggested by studies in the notum, in which it has been shown that the N receptor is distributed in a pattern complementary to Dl (Parks et al., 1997). If N levels were higher within the *hairy*-OFF vs. the *hairy*-ON interstripes in the leg, this could allow for preferential signaling within these domains. However, we assayed N expression in prepupal legs and found that N appears to be uniformly distributed along the leg circumference. Hence, either there is an asymmetric response to N or alternative mechanisms are responsible for establishing the directionality of Dl signaling in the leg, such as post-translational modification N signaling pathway components. For example, glycosylation of N by the Fringe glycosyltransferase influences its interactions with its ligands (Bruckner et al., 2000; Ju et al., 2000; Moloney et al., 2000; Munro and Freeman, 2000).

Another intriguing finding is the overlap of N signaling with the V-Hairy stripe. This result was surprising because it would suggest redundancy between *hairy* and Dl/N signaling in this region. However, we observe an absolute requirement for *hairy* function in the ventral leg. An explanation for this puzzling finding is suggested by the specific loss of the V-*Gbe+Su(H)m8-lacZ* stripe in *hairy* mutant legs, which indicates that Dl/N signaling or responsiveness in the ventral leg is dependent on *hairy* function. The specific loss of N signaling in the ventral leg could be a result of the expansion of *Dl* expression in *hairy* mutant legs, which as explained earlier might have a dominant-negative effect on N signaling. This proposal is corroborated by the expansion of *ac* expression along the circumference of legs ectopically expressing *Dl* throughout the tarsus. The overlap of *hairy* and Dl/N signaling in the ventral leg raises questions regarding the function of Dl/N signaling in this domain. We observed that V-*hairy* and *Gbe+Su(H)m8-lacZ* expression overlap only partially, suggesting that combined function of Dl and Hairy in the ventral leg could serve to establish a broader domain of repression in this region in comparison to other interstripe domains. This idea is supported by the morphology of the adult leg tarsus in which the spacing of bristles is most pronounced along the ventral midline (Hannah-Alava, 1958; Held, 1979). However, the function of N in the ventral leg is not as yet clear. It is plausible that there is a role for Dl/N signaling in the ventral leg that is unrelated to regulation of *ac* expression.

Distinct mechanisms are utilized to pattern the leg and notal microchaetae

The potential function of Dl as a regulator of proneural *ac* expression in the leg was suggested by studies in the notum, on which mechanosensory microchaetae are also organized in longitudinal rows (Parks et al., 1997). In the notum, Dl/Notch signaling, rather than Hairy, regulates periodic *ac* expression. Our studies suggest a distinct mechanism for leg microchaete patterning in which Hairy and Dl act together and nonredundantly to define periodic *ac* expression. In both the leg and notum, Dl signals to adjacent cells to repress *ac* expression. However, whereas in the notum Dl activates N signaling in cells on either side of each Dl/Ac stripe, in the leg, N signaling is activated (with one exception) only within the *hairy*-OFF interstripes. Although the pattern of mechanosensory bristles on the leg and notum is overtly similar, the bristle rows are more precisely aligned in the leg. The more organized pattern on the leg may be a consequence of the combined function of Hairy and Dl which might more precisely define the domains of proneural gene expression.

Regulation of Delta expression

We show that Dl function is essential for proper patterning of *ac* expression and suggest that accurate positioning of the Dl stripes is necessary for activation of Notch signaling within appropriate domains. Hence, regulation of *Dl* expression is an important aspect of leg microchaete patterning. In legs lacking *hairy* function, *Dl* expression expands into four broad domains and ectopic *hairy* expression greatly reduces *Dl* expression, indicating that periodic expression of *Dl* is regulated in part by *hairy*. Concomitant with the expansion of *Dl* expression, there is loss of N signaling in the ventral leg, suggesting that *hairy* functions to create an apposition of cells expressing high levels of Dl to cells expressing low levels of Dl, which allows for activation of N signaling in the ventral leg. Regulation of *Dl* expression in proneural fields is not understood. A plausible hypothesis is that, like *hairy*, *Dl* expression is established in response to the morphogens that control pattern formation during leg development.

Model for periodic patterning in the leg

This and previous studies allow us to outline a general genetic pathway for the regulation of *ac* expression in the leg microchaete proneural fields (Fig. 8A). This process involves broad and late activation, by an unknown factor, of *ac* expression along the leg circumference combined with refinement in response to a prepattern of repressors, which is established during larval and early prepupal stages. We have

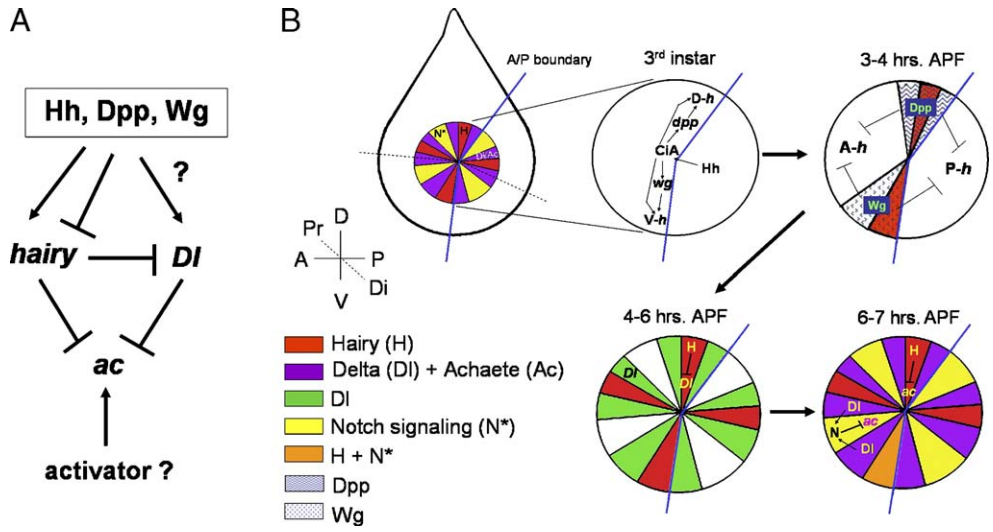

Fig. 8. Progressive patterning of leg microchaete bristles. (A) Pathway for regulation of *ac* expression in leg sensory bristle primordia. Global regulators Hh, Dpp and Wg establish spatially defined *hairy* and perhaps *Dl* expression. *ac* expression is uniformly activated along the leg circumference by an unknown activator, and its periodic expression is defined via repression by Dl and Hairy. Hairy also represses *Dl* expression. (B) Chronology of events which give rise to the longitudinal stripes of *hairy*, *ac* and *Dl* expression in the tarsus of prepupal legs at 6 h APF. The circles represent the tarsus (individual tarsal segments are not depicted). The stripes of Hairy, Dl and Ac are superimposed on a diagram of a 3rd instar leg disc. The D/V-*hairy* stripes are activated by Hh, Dpp and Wingless during the 3rd larval instar. A/P-*hairy* expression begins between 3 and 4 h APF. Genetic studies suggest that they are activated uniformly, by an unknown mechanism, and are repressed dorsally and ventrally by Dpp and Wg, respectively. *Dl* expression within the mechanosensory microchaete primordia is established between 4 and 6 h APF. Hairy, which is expressed in four longitudinal stripes by 4 h APF, represses *Dl* expression, resulting in 8 longitudinal stripes of Dl, which overlap the microchaete proneural fields. Shortly after the Dl stripes have been established, *ac* expression is activated uniformly along the leg circumference but is confined to the proneural stripes via Hairy- and Delta-mediated repression. By 6 h APF, a prepattern of repression is established in which *hairy* is expressed in four Ac interstripes and N signaling is activated in the complementary set of Ac interstripes. Hairy expression and N signaling overlap only in the ventral leg, as indicated.

identified Hairy and Dl as the primary prepattern factors that regulate *ac* expression along the leg circumference. Position-specific expression of both *hairy* and *Dl* in longitudinal stripes is essential for proper *ac* expression. We have determined that the longitudinal stripes of *hairy* are established in direct response to the Hh, Dpp and Wg signals, which globally pattern the leg, indicating that *hairy* acts as an interface between *ac* and these morphogens. *Dl* expression is regulated by Hairy, but its regulation is otherwise poorly understood. In addition to elucidating a pathway for establishment of periodic *ac* expression during leg development, these studies also provide insight into the mechanisms through which morphogens function to generate leg morphology.

Periodic *ac* expression is established progressively as shown in Fig. 8B. The first evidence of periodicity is expression of the longitudinal stripes of *hairy* expression. The D/V-*hairy* stripes are expressed first in the early 3rd instar leg disc followed by the A/P-stripes between 3 and 4 h APF. Between 4 and 6 h APF, *Dl* expression within the mechanosensory microchaete primordia is established. Then, *ac* expression is activated uniformly along the leg circumference. By the time that *ac* expression is activated, the interstripe domains have been defined by the four Hairy stripes and Dl/N signaling.

The delay of *ac* expression in the microchaete proneural fields until mid-prepupal stages is likely due to the requirement of *ac* function for formation of all leg sensory organs. Leg sensory bristles can be grouped into two broad categories based on their time of specification: one group includes the early-specified mechanosensory macrochaetae (large bristles) and chemosensory microchaetae, and the second group includes the more numerous late-specified mechanosensory microchaetae. During the 3rd instar and early prepupal stages, *ac* is expressed in small clusters of cells that define the primordia of early-specified bristles, while expression of *ac* in the mechanosensory microchaete primordia is activated later in the mid-prepupal stage. This late expression of *ac* is activated broadly along the leg circumference and is presumably delayed to allow for expression of the *hairy* and *Dl* stripes during earlier stages. Premature expression of this normally late *ac* expression would likely lead to disturbances in sensory organ patterning, suggesting that temporal control of *ac* expression is an important aspect of its regulation.

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