# Opposing inputs by Hedgehog and Brinker define a stripe of *hairy* expression in the *Drosophila* leg imaginal disc

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## Summary

**The sensory organs of the *Drosophila* adult leg provide a simple model system with which to investigate pattern-forming mechanisms. In the leg, a group of small mechanosensory bristles is organized into a series of longitudinal rows, a pattern that depends on periodic expression of the *hairy* gene (*h*) and the proneural genes *achaete* (*ac*) and *scute* (*sc*). Expression of *ac* in longitudinal stripes in prepupal leg discs defines the positions of the mechanosensory bristle rows. The *ac*/*sc* expression domains are delimited by the Hairy repressor, which is itself periodically expressed. In order to gain insight into the molecular mechanisms involved in leg sensory organ patterning, we have analyzed a Hedgehog (Hh)- and Decapentaplegic (Dpp)-responsive enhancer of the *h* gene, which directs expression of *h* in a narrow stripe in the dorsal leg imaginal disc (the D-*h* stripe). Our studies suggest that the domain of D-*h* expression is defined by the overlap of Hh and high-level Dpp signaling. We find that the D-*h* enhancer consists of a Hh-responsive activation element (HHRE) and a repression element (REPE), which responds to the transcriptional repressor Brinker (Brk). The HHRE directs expression of *h* in a broad stripe along the anteroposterior (AP) compartment boundary. HHRE-directed expression is refined along the AP and dorsoventral axes by Brk1, acting through the REPE. In D-*h*-expressing cells, Dpp signaling is required to block Brk-mediated repression. This study elucidates a molecular mechanism for integration of the Hh and Dpp signals, and identifies a novel function for Brk as a repressor of Hh-target genes.**

Key words: *hairy*, *hedgehog*, *decapentaplegic*, *brinker*, Mechanosensory organs, Leg imaginal disc development, *Drosophila*

## Introduction

Pattern formation in vertebrates and invertebrates is controlled by several conserved signaling molecules that cooperate to specify particular cell fates at different threshold concentrations (Gurdon and Bourillot, 2001; Tabata, 2001). Crucial to this process are the cellular responses that give rise to the exquisitely patterned body plans of multicellular organisms. One important response to signals is the establishment of position-specific expression of genes that function at a local level to specify cell fates. The *Drosophila melanogaster* adult leg provides a simple model system with which to investigate this process. On the surface of the *Drosophila* leg, a group of small mechanosensory bristles, called microchaetae, are organized in a series of longitudinal rows around the leg circumference. The orderly arrangement of the leg microchaetae depends largely on the periodic expression of just a few genes, including the antineural gene, *hairy* (*h*), and the redundant proneural genes, *achaete* (*ac*) and *scute* (*sc*) (Orenic et al., 1993). Hence, mechanistic insight into the periodic patterning of leg microchaetae can be gained by investigating the regulation of *h* and *ac/sc* expression.

Extensive studies on the development of sensory structures in the *Drosophila* mesothorax and other tissues have shown that the redundant proneural genes, *ac* and *sc*, function at a local level to confer neural competence to cells destined to become sensory organs (SOs) (Calleja et al., 2002; Modolell, 1997). The bHLH transcription factors, Ac and Sc, are expressed in proneural clusters, groups of cells that roughly define the positions of future sensory structures in the adult (Cubas et al., 1991; Romani et al., 1989; Skeath and Carroll, 1991). Then, through local regulatory events controlled by the neurogenic genes, a cell(s) is selected from each proneural cluster to become a sensory organ precursor, which undergoes a few differential cell divisions (Calleja et al., 2002; Modolell, 1997). The resulting cells give rise to the components of the SO. In prepupal legs, expression of *ac* and *sc* in a series of longitudinal proneural stripes around the leg circumference defines the primordia of the mechanosensory microchaetae. Expression of *ac* in the leg is regulated by *h* (Orenic et al., 1993), which is itself periodically expressed in two pairs of longitudinal stripes, one pair that traverses the dorsoventral (DV) axis (DV-*h*) and another pair that runs along the anteroposterior (AP) axis (AP-*h*) (Carroll and Whyte, 1989; Hays et al., 1999). On either side of each *h* domain, a stripe of *ac* expression demarcates the position of each leg microchaete bristle row (Orenic et al., 1993). *h* encodes a bHLH

transcription factor (Rushlow et al., 1989) and is a direct repressor of *ac* expression (Ohsako et al., 1994; Van Doren et al., 1994). In the absence of *h* 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. Therefore, precise position-specific expression of *h* in leg discs is crucial for generation of the adult leg bristle row pattern.

We have investigated the regulation of two *h* stripes, the DV-*h* stripes, and have found that they are established in response to the signaling molecules that globally pattern leg imaginal discs (Hays et al., 1999). Expression of the DV-*h* stripes is controlled by a pair of modular enhancers that direct expression of the dorsal (D-*h*) and ventral (V-*h*) stripes, respectively (Fig. 1). Here, we focus on the function of the D-*h* stripe enhancer, which directs expression of *h* in a narrow dorsal domain positioned a few cells anterior to the AP-compartment boundary and integrates input from the Hedgehog (Hh) and Decapentaplegic (Dpp) signaling pathways.

In leg imaginal discs, Hh secreted by posterior compartment cells signals to anterior cells to activate expression of *dpp* in a primarily dorsal stripe near the AP boundary (Diaz-Benjumea et al., 1994). Hh activates gene expression through its transcriptional mediator Cubitus interruptus (Ci), a zinc-finger transcription factor (Alexandre et al., 1996; Dominguez et al., 1996; Hepker et al., 1997; Von Ohlen et al., 1997). Ci is expressed specifically in anterior compartment cells and exists in either a cleaved repressor or a full-length activator form (Aza-Blanc et al., 1997). In cells near the AP boundary, Hh prevents cleavage and promotes nuclear entry of the full-length form of Ci, allowing activation of Hh-target genes, such as *dpp* (Aza-Blanc et al., 1997; Chen et al., 1999; Methot and Basler, 1999; Methot and Basler, 2000; Wang and Holmgren, 1999; Wang and Holmgren, 2000).

Dpp acts as a long-range morphogen and regulates gene expression in imaginal discs in a concentration-dependent manner (Lecuit et al., 1996; Nellen et al., 1996). In response to Dpp signaling, an activated form of the Smad transcription factor, Mothers against dpp (Mad), is generated. Mad then binds to a related protein, Medea (Med), and this complex translocates to the nucleus to transcriptionally regulate expression of Dpp target genes (Raftery and Sutherland, 1999; Zimmerman and Padgett, 2000). In a number of cases, it has been observed that the Mad/Med complex binds the enhancers of Dpp target genes and directly activates transcription (Kim et al., 1997; Rushlow et al., 2001; Szuts et al., 1998). However, more recent studies indicate that Dpp also regulates its target genes by blocking expression of a repressor. In imaginal discs, Mad/Med and the zinc-finger protein Shn, repress expression of the *brinker* (*brk*) gene (Marty et al., 2000; Muller et al., 2003; Torres-Vazquez et al., 2000), which encodes a direct repressor of Dpp target genes (Campbell and Tomlinson, 1999; Jazwinska et al., 1999; Kirkpatrick et al., 2001; Minami et al., 1999; Rushlow et al., 2001; Saller and Bienz, 2001; Sivasankaran et al., 2000; Zhang et al., 2001). The Brk repressor is distributed in a gradient reciprocal to Dpp signaling and functions to delimit the domains of Dpp target gene expression. It is thought that competing interactions between Brk and Mad/Med define the expression domains of some Dpp-target genes (Kirkpatrick et al., 2001; Rushlow et al., 2001; Saller and Bienz, 2001).

Our genetic studies suggest that Hh and Dpp signaling are both required to activate D-*h* expression (Hays et al., 1999). Based on these genetic observations, we proposed that Ci and Mad/Med act synergistically to activate *h* expression in the dorsal region of the leg disc. To test this model and to investigate the molecular mechanisms underlying integration of the Hh and Dpp signals, we have undertaken a molecular analysis of the D-*h* enhancer. We show that the D-*h* enhancer consists of at least two distinct sub-elements: a Hh-responsive element (HHRE), which directs expression in a broad AP boundary stripe, and a repression element (REPE), which refines HHRE-directed expression along the AP and DV axes. We find that Brk acts through the REPE to repress HHRE-directed expression, while Dpp functions to block Brk-mediated repression in a narrow dorsal stripe. These observations suggest a novel role for Brk in repression of a Hh-target gene and elucidate a mechanism for the integration of the Hh and Dpp signals. Furthermore, this study establishes a correlation between enhancer function and the formation of specific morphological elements, the dorsal microchaete bristle rows of the adult leg.

## Materials and methods

### Fly strains and genetics

The following fly strains were used in this study: *smo*$^{IIG26}$ FRT40A/CyO (Blair and Ralston, 1997), *dpp*$^{d6}$/CyO, *dpp*$^{d12}$/CyO (Spencer et al., 1982), *w*; *wg*$^{Sp-1}$/CyO; *dpp*$^{blk}$-*Gal4* /TM6B, Tb1 (Staehling-Hampton et al., 1994), *y w brk*$^{38-3}$ *f*$^{36a}$ FRT18A/FM7 (Minami et al., 1999), UAS-*brk*, *brk-lacZ* (Jazwinska et al., 1999), UAS-*tkv*$^{Q253D}$ (Nellen et al., 1996) and D-*h-lacZ* (Hays et al., 1999). *smo*$^{IIG26}$ is a strong hypomorphic mutation (van den Heuvel and Ingham, 1996). The *dpp*$^{d6}$/CyO, *dpp*$^{d12}$/CyO, *w*; *wg*$^{Sp-1}$/CyO, *dpp-Gal4*/*TM6B, Tb1*, *dpp-lacZ*, UAS-*GFP* and *FRT* strains were obtained from the Bloomington Stock Center. The following transgenic lines were generated for this study (see below for details): D-*h-GFP*, D-*h*-Ci1-*lacZ*, D-*h*-MM-*GFP*, D-*h*-MBM-*GFP*, D-*h*-C-*GFP*, D-*h*-CB-*GFP*, D-*h*-MCM-*GFP*, D-*h*-M2-*GFP*, D-*h*-BM2-*GFP*, HHRE-*lacZ*, HHRE-*GFP*, HHRE-Ci1-*lacZ*, HHRE-Ci2 and HHRE-Ci1+2-*lacZ*.

*smo* and *brk* mutant clones were made in larvae of the genotypes: *y hs-flp*/HHRE-*GFP*; *smo*$^{IIG26}$ FRT40A/*hs-piMyc36F* FRT40A or *brk* FRT18A/*hs-piMyc*10D FRT18A; D-*h-GFP*/+; *hs-flp*/+. Clones were generated by heat shocking larvae (48-96 hours after egg laying, AEL) for 1 hour at 37°C. Prior to dissection, third instar larvae or prepupae were heat shocked for 1 hour at 37°C to induce *piMyc* expression and were then allowed to recover for 1 hour.

For analysis of transgene expression in leg discs with reduced *dpp* function, discs were dissected from larvae or prepupae of the following genotypes: (1) D-*h-GFP*; *dpp*$^{d6}$/*dpp*$^{d12}$, (2) HHRE-*GFP*/; *dpp*$^{d6}$/*dpp*$^{d12}$ and (3) *brk-lacZ*; *dpp*$^{d6}$/*dpp*$^{d12}$. For ectopic expression studies, leg imaginal discs were dissected from larvae or prepupae of the following genotypes: (1) D-*h-GFP*;UAS-*tkv*$^{Q253D}$/+; *dpp-Gal4*/+, (2) D-*h-GFP*; UAS-*brk*/+; *dpp-Gal4*/+, (3) HHRE-*GFP*; UAS- *brk*/+; *dpp-Gal4*/+, (4) D-*h*-CB-*GFP*; UAS-*brk*/+; *dpp-Gal4* and (5) D-*h*-C-*GFP*; UAS-*brk*/+; *dpp-Gal4*.

### Immunohistochemistry and microscopy

For anti-Myc (Xu and Rubin, 1993), anti-β-galactosidase (Promega) and anti-Engrailed (Patel et al., 1989) staining, dissected imaginal discs or prepupal legs were treated as described (Carroll and Whyte, 1989). All images were collected on a Zeiss Axiovert 200 M equipped with a digital camera. Fluorescent images were collected as *z*-stacks and were subjected to 3D-deconvolution.

### Generation of wild-type and mutant reporter constructs and transformation

To generate the *lacZ*-transgenes, the corresponding wild-type and mutant genomic DNA fragments were cloned in HSPCasper-*lacZ* (Nelson and Laughon, 1990). *GFP* transgenes were generated by cloning the corresponding wild-type and mutant genomic DNA into the pHStinger vector (Barolo et al., 2000). The pHStinger vector offers the advantage that the reporter gene is flanked by insulator sequences from the gypsy transposon. The insulator sequences reduce position effects and thus result in less variability in expression among different transgenic lines. Expression was assayed from at least three independent transgenic lines for each construct; similar expression levels were observed among all the lines. In addition, similar results were obtained with insertions in both vectors. The D-*h* transgenes were generated by cloning a 3.4 kb *Bam*HI/*Eco*RI genomic fragment (see Fig. 1B and results) into the HSPCasper or pHStinger vectors. A 1 kb *Bam*HI/*Hin*dIII subfragment (Fig. 1B) from the D-*h* enhancer was cloned into the corresponding vector to generate the HHRE (Hh response element, see results) transgene.

All the site mutations were generated using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) or the USE Mutagenesis Kit (Pharmacia) and are as follows (altered bases are shown in lower case).

Ci binding site mutations in the HHRE: Wild type, GACCTCCCA……..GACCACCAT; Ci1, GACCTCCCA…….. GAgttCCAT; Ci2, GACaTCCCA……..GACCACCAT; and Ci1+2, GACaTCCCA……..GACaACCAT.

CMB site mutations in the D-*h* enhancer: Wild type, GCGACGGCGTCATC; CRE(C), GCGACGGCGcCgTC; Mad1/CRE/-Mad2(MCM), aattCGGCGcCtTt; Mad1/Brinker/Mad2(MBM), aattCGaCGTCATt; CRE/Brinker(CB), GCGACGGCGaCtgC; Mad1/Mad2(MM), aattCGGCGTCAag; Mad2(M2), GCGACGGCGTCAag; Brinker/Mad2(BM2), GCGACGaCGTCAag.

Prior to introducing the mutated fragments into flies, all the mutagenized regions were tested by gel mobility shift assays with Ci zinc-finger domain, Brinker and/or N-Mad proteins. Several versions of the MM mutant were tested; all affected Brk as well as Mad binding. The version used in this study had the least effect on Brk binding, while still reducing Mad binding to a significant degree. Reporter genes were introduced into flies by P-element-mediated germline transformation (Rubin and Spradling, 1982).

### Protein preparations and gel mobility shift assays

The Ci DNA-binding domain was prepared using the TNT Coupled Reticulocyte System (Promega) as described (Hepker et al., 1999). The GST-N-Mad (Kim et al., 1997) was induced in *E. coli* BL21 with 100 mM IPTG. The cells were harvested by centrifugation and sonicated on ice. After incubating with Triton X-100 to 1% for 30 minutes, the lysate was pelleted by centrifugation, and the supernatant was used to purify the proteins with the GSTrap column (Amersham Pharmacia Biotech). Brinker protein was prepared in the TNT Coupled Reticulocyte System, from a *brk* cDNA (Minami et al., 1999), which was cloned into the *Sma*I site of pGEM4Z (Promega) as *Dra*I fragment.

For gel mobility shift assays, oligonucleotide probes were end-labeled with [$\alpha$-$^{32}$P]dCTP (3000 Ci/mmol, ICN) and purified with a Sephadex spin column. Prepared proteins were incubated on ice for 30 minutes with ~50,000 c.p.m. of labeled probes in binding buffers as described (Hepker et al., 1999; Kim et al., 1997; Sivasankaran et al., 2000). In some samples, unlabelled wild-type oligos (specific competitor) and mutant oligos (nonspecific competitor) were included. The mixtures were separated in 5% polyacrylamide gels in 0.5×TBE.

The following oligos were used for gel mobility shift assays (top strands are shown). Ci1 wild type, CTGAATGGAGGACCA-CCATGTGTGT; Ci1 mutant, CTGAATGGAGGACaACCATGTG-TGT; Ci2 wild type, CCAGCCATCCGACCTCCCAACCATT; Ci2 mutant, CCAGCCATCCGACaTCCCAACCATT; Mad/Brinker wild type, GCTTTTCGGCGACGGCGTCATCTTGTCATC; Mad-double mutant, GCTTTTCGagatCGGCGTCAaaTTGTCATC; Mad-single mutant, GCTTTTCGGCGACGaCGTCAaaTTGTCATC; Brinker mutant, GCTTTTCGGCGACaattaaATCTTGTCATC; CRE-mutant, GCTTTTCGGCGACGGCGcCgTCTTGTCATC; Mad2-mutant, GC-TTTTCGGCGACGGCGTCAagTTGTCATC; Brk/Mad2-mutant, GC-TTTTCGGCGACGaCGTCAagTTGTCATC; Mad1/Mad2-mutant, GCTTTTCGaattCGGCGTCAagTTGTCATC.

## Results

In a previous study, we reported that the D-*h* and V-*h* stripes are regulated by separate enhancers, which map between 32-38 kb 3′ to the *h* transcription unit (Hays et al., 1999). Expression of the DV-*h* stripes relative to their flanking *ac* stripes and the compartment boundary is depicted in Fig. 1A. Although the *ac* stripes are not expressed until 6 hours after puparium formation (APF), they are projected onto a third instar leg disc for the sake of clarity. The narrow D-*h* stripe is

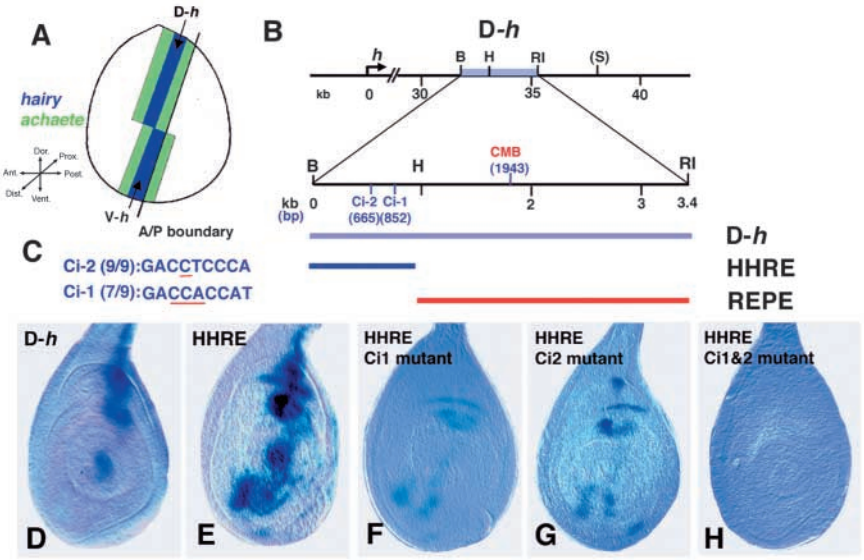



**Fig. 1.** D-*h* expression is activated by a Hh-response element. (A) A third instar leg imaginal disc depicting expression of the DV-*h* stripes (blue) relative to *ac* expression (green) and the compartment boundary. The D-*h* and V-*h* stripes occupy distinct registers relative to the AP boundary. (B) (Top) map of the *h* locus showing the position of the D-*h* enhancer. Below is shown a more detailed map of the D-*h* enhancer. Fragments tested in reporter constructs are shown beneath the detailed map: full-length D-*h* enhancer (light blue) and the Hh response element (HHRE, dark blue), which has two Ci binding sites and the repression element (REPE, red; see text and Fig. 3). The CMB site in the REPE is described in the text and in Fig. 8. (C) Sequence of two Ci-binding sites present in the HHRE. Underlined bases were altered in the Ci1, Ci2 or Ci1&2 double mutant (see Materials and methods). (D,E) *lacZ* expression in leg imaginal discs carrying: D-*h*-*lacZ* (D) and HHRE-*lacZ* (E). (F-G) *lacZ* expression directed by HHRE-*lacZ* site mutants: Ci1 mutant (F), Ci2 (G) and Ci1&2 (H).

positioned a few cells anterior to the boundary, allowing expression of two dorsal *ac* stripes in the anterior compartment. V-*h*, however, is expressed directly adjacent to the AP boundary so that there is only one ventral *ac* stripe in the anterior compartment. Expression of each *h* stripe in its proper register is essential for positioning of the *ac* stripes and consequently for sensory bristle patterning in the adult leg. We focus on the mechanisms that lead to expression of the D-*h* stripe in its precise register near the AP boundary.

### A Hh responsive activation element is required for D-*h* enhancer activity

We previously showed that expression of the endogenous D-*h* stripe is dependent on Hh signaling (Hays et al., 1999). In order to identify sequences that mediate Hh responsiveness, we undertook a dissection of the D-*h* enhancer. The D-*h* enhancer maps to a 3.4 kb *Bam*HI/*Eco*RI fragment located 32 kb 3′ to the *h* structural gene (Fig. 1B). In third instar leg imaginal discs, this fragment directs *lacZ* expression in a dorsally restricted AP boundary-adjacent stripe (Fig. 1D). Two subfragments of the D-*h* enhancer were tested for the ability to drive reporter gene expression in leg imaginal discs. A 3′ 2.4 kb *Hind*III/*Eco*RI subfragment of the D-*h* enhancer (REPE in Fig. 1B) directs no detectable reporter gene expression in leg imaginal discs (not shown). However, the complementary 5′ 1.0 kb *Bam*HI/*Hin*dIII fragment of the D-*h* enhancer drives expression in a stripe that is not dorsally restricted but rather traverses the entire length of the DV axis (Fig. 1E), suggesting it responds to Hh signaling in both dorsal and ventral leg cells. To determine whether Hh signals through the *Bam*HI/*Hin*dIII fragment of the D-*h* enhancer, expression from a *Bam*HI/*Hin*dIII-*GFP* transgene was assayed in leg clones lacking function of Smoothened (Smo), a transmembrane protein required for transduction of the Hh signal (Alcedo et al., 1996; van den Heuvel and Ingham, 1996). Somatic clones lacking *smo* function were generated by FLP/FRT-mediated mitotic recombination (Xu and Rubin, 1993). We observed cell-autonomous loss of *GFP* expression in *smo* clones that overlapped the GFP stripe (Fig. 2A-C). These observations imply that Hh signals through the *Bam*HI/*Hin*dIII fragment, and therefore, we refer to this region as the D-*h*-Hh response element (HHRE).

As the HHRE is Hh responsive, we searched the element for the consensus binding site of the Hh pathway transcriptional effector, Ci (Alexandre et al., 1996; Dominguez et al., 1996; Hepker et al., 1997; Von Ohlen et al., 1997). Two potential Ci-binding sites (Ci-1 and Ci-2) were found, each of which matches the consensus, TGGG(A/T)GGTC (Zarkower and Hodgkin, 1993), in a minimum of seven out of nine sites (Fig. 1C) and bind the Ci zinc-finger domain (CiZn) in a electrophoretic mobility shift assay (EMSA) (see Fig. 3, lanes 1-7 show sequence-specific binding to the Ci-1 site). To determine whether the Ci-binding sites are required for HHRE-directed expression, point mutations were introduced into the Ci-1 and 2 sites (Fig. 1C). These mutations abolished Ci binding of the HHRE in vitro (Fig. 3, lane 7, Ci-1 mutant shown). Expression directed by the HHRE with a mutation in either the Ci-1 or Ci-2 sites is drastically compromised (Fig. 1F,G), and there is no detectable expression from an HHRE-*lacZ* transgene with both Ci sites mutated (Fig. 1H). Taken together, these studies indicate that D-*h* expression is activated primarily by the HHRE, through which Ci acts as an essential and direct transcriptional activator.

### A D-*h* enhancer repression element restricts Hh-mediated activation of D-*h*

The D-*h* enhancer directs expression of a narrow GFP stripe, which coincides with endogenous D-*h* expression (Fig. 4A-C). HHRE-directed expression, however, is broader not only along the DV axis as observed in Fig. 1E (see also Fig. 5B) but also along the AP axis. Fig. 4D,E shows that the HHRE-GFP stripe extends a few cells anterior and posterior to the endogenous D-*h* stripe. These observations imply that sequences in the 3′ *Hind*III/*Eco*RI fragment of the D-*h* enhancer (Fig. 1B) function to repress ventral D-*h* expression and to restrict stripe width along the AP axis. We, therefore, refer to the *Hind*III/*Eco*RI fragment as the D-*h*-repression element (REPE).

It appears, then, that the spatial domain of D-*h* expression is established through broad activation by a Hh-responsive element and refinement by an associated repressive element, the REPE. This refinement is presumably necessary to maintain the proper register of D-*h* expression relative to the compartment boundary, allowing proper positioning of the dorsal *ac* stripes and consequently the leg bristle rows. The register of D-*h*-GFP and HHRE-GFP stripes relative to *engrailed* (*en*) expression is shown in Fig. 4G-J. Note that the D-*h*-GFP stripe is positioned a few cells anterior to the compartment boundary, while HHRE-*GFP* expression extends up to the compartment boundary [the overlap between HHRE-*GFP* and *en* expression is due to low-level anterior compartment *en* expression which is observed beginning at the mid-third instar in wing discs (Blair, 1992) and after puparium formation in leg discs (Inaki et al., 2002)].

### Dpp signaling is required to block repression of D-*h* expression

We have previously shown that endogenous D-*h* expression is compromised in somatic clones lacking function of Mad, the transcriptional effector of Dpp signaling, and that D-*h*-*lacZ* expression is severely decreased in leg imaginal discs with reduced *dpp* function (Hays et al., 1999). Furthermore, D-*h*-*lacZ* expression is ventrally expanded in *wingless* (*wg*) mutant

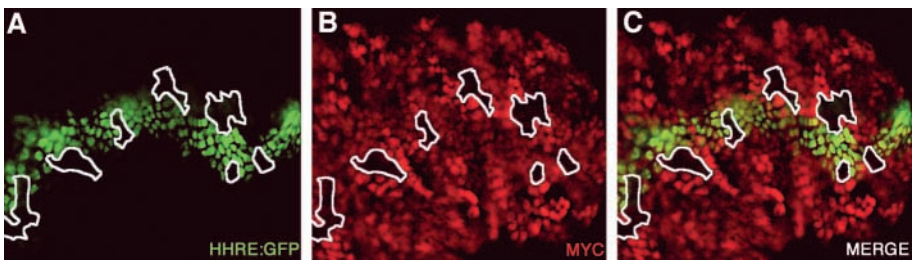
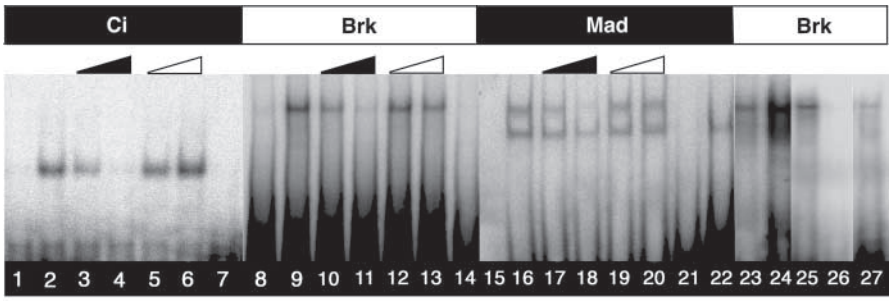



**Fig. 2.** Hh signaling is required for function of the D-*h* activation element. Clones lacking *smo* function were examined in a prepupal leg, 3 hours APF. (A) HHRE-*GFP* expression (green). (B) *myc* expression (red). Homozygous $smo^{IIG26}$ clones are identified by the lack of Myc expression. (C) Merge of images in A and B (several *smo*- clones that overlap the HHRE-GFP stripe are outlined in white). Note the absence HHRE-*GFP* expression in $smo^{-}$ clones




| Probe | Sequence |
|---|---|
| Ci1 wt. | CTGAATGGAGGACCACCATGTGTGTGCCA |
| Ci1 mut. | CTGAATGGAGGACaACCATGTGTGTGCCA |
| CMB wt. | GCTTTTCGGCGACGGCGTCATCTTGTCATC |
| Brk mut. | GCTTTTCGGCGACaattaaATCTTGTCATC |
| Mad dmut. | GCTTTTCGagatCGGCGTCAaaTTGTCATC |
| Mad smut. | GCTTTTCGGCGACGaCGTCAaaTTGTCATC |
| C mut. | GCTTTTCGGCGACGGCGcCgTCTTGTCATC |
| M2 mut. | GCTTTTCGGCGACGGCGTCAagTTGTCATC |
| BM2 mut. | GCTTTTCGGCGACGaCGTCAagTTGTCATC |
| MM mut. | GCTTTTCGaattCGGCGTCAagTTGTCATC |

**Fig. 3.** Ci binds the D-*h* activation element, while Brk and Mad bind the repression element. Binding of the Ci DNA-binding domain to the Ci1 site in HHRE (lanes 1-7) and binding of full-length Brk or GST-N-Mad protein to the CMB element (lanes 8-27) were tested by EMSA. Sequences of Ci1 site wild-type (Ci1 wt), CMB site wild-type (CMB wt) and the following mutant probes are shown below: Ci1 site mutant (Ci1 mut), Brk site mutant (Brk mut), Mad site double-mutant (Mad dmut), Mad site single-mutant (Mad smut), CRE mutant (C mut), Mad2 site mutant (M2 mut), Brk/Mad2 site mutant (BM2 mut) and Mad1/Mad2 mutant (MM mut). Putative binding sites are underlined on the wild-type sequences. Lanes 1-7, sequence-specific binding of Ci protein to the Ci1 site; lane 1, Ci1 wild-type probe and no protein; lanes 2-6, Ci1 wt probe + Ci protein and no competitor (lane 2), 10× specific competitor (Ci1 wt oligo) (lane 3), 100× specific competitor (lane 4), 10× nonspecific competitor (Ci1 mut oligo) (lane 5), and 100× nonspecific competitor (lane 6). Lane 7, Ci1 mut oligo + Ci protein; lanes 8-14, sequence-specific binding of Brk protein to the CMB element; lane 8, CMB wt probe and no protein; lanes 9-13, CMB wt probe + Brk protein and no competitor (lane 9), 10× specific competitor (CMB wt oligo) (lane 10), 100× specific competitor (lane 11), 10× nonspecific competitor (Brk mut oligo) (lane 12), 100× nonspecific competitor (lane 13). Lane 14, Brk mut probe + Brk protein; lanes 15-22, sequence specific binding of Mad protein to the CMB element; lane 15, CMB wt probe and no protein; lanes 16-22, CMB wt probe + Mad protein and no competitor (lane 16), 10× specific competitor (CMB wt oligo) (lane 17), 100× specific competitor (lane 18), 10× nonspecific competitor (Mad dmut oligo) (lane 19), 100× nonspecific competitor (lane 20). Lane 21-22, Mad protein + Mad dmut probe (lane 21) or Mad smut probe (mutation is in Mad2 site) (lane 22). Lanes 23-27, Brk protein binding to CMB wt probe (lane 23), C mut probe (lane 24), M2 mut probe (lane 25), BM2 mut probe (lane 26) and MM mut (lane 27).

legs, which have strong ventral *dpp* expression (Brook and Cohen, 1996; Jiang and Struhl, 1996; Johnston and Schubiger, 1996; Morimura et al., 1996; Penton and Hoffmann, 1996; Theisen et al., 1996). These findings indicate a requirement for Dpp, in addition to Hh signal, for D-*h* expression. The most parsimonious model to explain how *h* integrates positive input from the Hh and Dpp signals, is that Mad acts synergistically with Ci through the D-*h* enhancer to activate D-*h* expression. However, we show here that Dpp is instead required to block REPE-mediated repression.

As shown above, the HHRE directs expression in a broad stripe that extends into the ventral leg (Fig. 1E, Fig. 5B), where there is little or no Dpp signaling, implying that HHRE-directed expression does not require Dpp function. To determine whether HHRE-directed expression is Dpp independent, D-*h*-*GFP* and HHRE-*GFP* expression were examined in $dpp^{d6}/dpp^{d12}$ leg imaginal discs, which have reduced *dpp* function. In these leg discs, D-*h*-*GFP* expression is severely compromised (compare Fig. 5A with 5F), while HHRE-*GFP* expression is unaffected (compare Fig. 5B with 5G; note that in both *dpp* mutant and wild-type leg discs, the HHRE-GFP stripe is 5-6 cells wide). This result suggests that Dpp signals through the REPE. As the REPE functions as a repressive element, Dpp probably functions to block the repressive effects of this element rather than to activate D-*h* expression.

In leg discs, *dpp* is expressed in a broad AP boundary adjacent stripe, which is stronger dorsally than it is ventrally

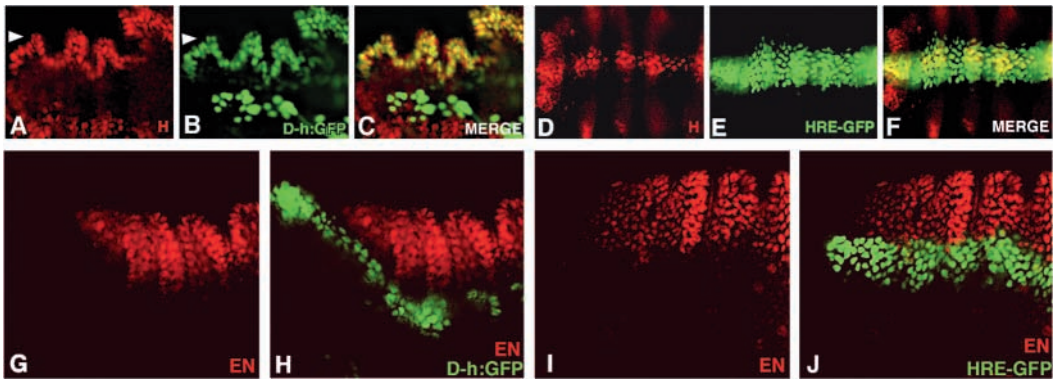



**Fig. 4.** A D-*h* repression element attenuates activity of the activation element. (A-F) Comparison of endogenous Hairy [red in A,D (arrowhead indicates endogenous D-*h* stripe)] with D-*h*-*GFP* [green in B (arrowhead indicates D-*h*-GFP stripe)] and HHRE-*GFP* (green in E) expression in prepupal legs, 3 hours APF. (C) Merge of images in A and B. (F) Merge of images in D and E. *en* expression (red in G-J) relative to D-*h*-*GFP* (green in H) and HHRE-*GFP* (green in J) in prepupal legs 5 hours APF.

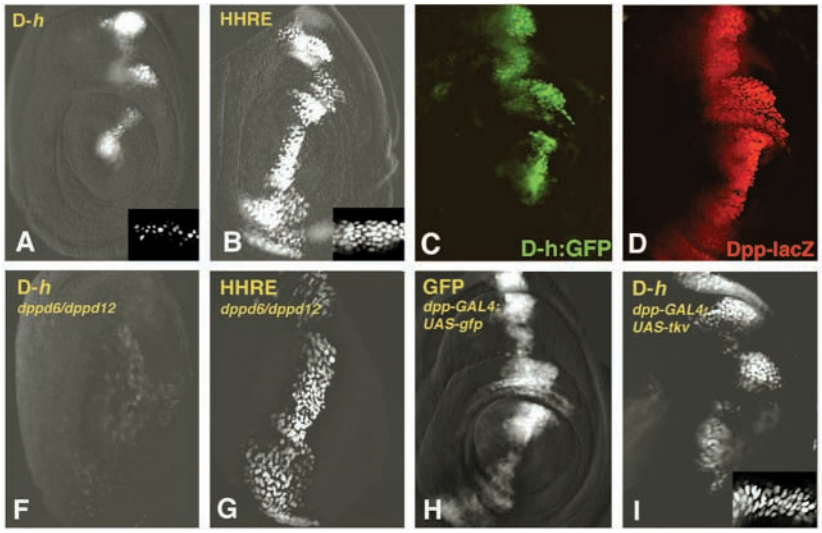



**Fig. 5.** Dpp signals through a D-*h* repression element and defines the domain of D-*h* expression. D-*h*-*GFP* (A) and HHRE-*GFP* expression (B) in wild-type leg imaginal discs. The insets in A, B and I show *GFP* expression in the basitarsal segment of a 4-hour prepupal leg. (C-E) Comparison of D-*h*-*GFP* expression (C) with *dpp-lacZ* expression (D). (E) Merge of images in C and D. (F,G) In legs with reduced *dpp* function (*dppd6/dppd12*), D-*h*-*GFP* expression (F) is significantly compromised, while HHRE-*GFP* expression appears normal (G). (H) *GFP* expression in a UAS-*GFP*/+; *dpp-Gal4*/+ leg imaginal disc. (I) In UAS-$tkv^{QD}$/+; *dpp-Gal4*/+ legs, Dpp signaling is elevated within *dpp*-expressing cells, resulting in partial ventral expansion and widening of the D-*h*-GFP stripe.

(see *dpp-lacZ* expression, Fig. 5D). D-*h*-*GFP* expression coincides with a subset of cells expressing the highest levels of *lacZ* within the *dpp* stripe (Fig. 5C-E). Perhaps, then, high-level Dpp signaling functions, by overcoming repressive effects of the REPE, to define a narrow stripe of D-*h* expression within a broader region defined by Hh. A prediction stemming from this hypothesis is that elevation of Dpp signaling within the HHRE-response zone would expand D-*h* expression. To test this premise, Dpp signaling was elevated along the AP boundary by expression of a constitutively active form of the Dpp receptor Thickveins ($Tkv^{QD}$) (Nellen et al., 1996). A *dpp-Gal4* transgene (Staehling-Hampton et al., 1994), which drives *Gal4* expression in a broad AP boundary stripe at high levels in the dorsal leg and more weakly in the ventral leg (Fig. 5H), was used to express UAS-$tkv^{QD}$. This results in broadening of the D-*h*-GFP stripe and partial expansion into the ventral disc (compare Fig. 5A with 5I). The insets in Fig. 5 show D-*h*- and HHRE-GFP expression in the basitarsal segment of leg discs dissected at 4 hours APF. By this time, the tarsal segments are obviously separated and partially extended, allowing for accurate measurement of the breadth of each GFP stripe. Note that the D-*h* stripe is two cells wide (Fig. 5A), while in the leg expressing $tkv^{QD}$, D-*h*-*GFP* expression is four or five cells wide, similar to the HHRE-GFP stripe (Fig. 5B). Together these studies support the hypothesis that high-level Dpp signaling defines the position of the D-*h* stripe within the Hh response zone by interfering with REPE function.

## Brinker opposes Hh signaling through the D-*h* enhancer

Studies so far raise a question regarding the identity of the repressor(s) that acts through the REPE to refine HHRE-directed expression. A potential candidate, the transcriptional repressor of Dpp target genes, Brk (Campbell and Tomlinson, 1999; Jazwinska et al., 1999; Kirkpatrick et al., 2001; Minami et al., 1999; Rushlow et al., 2001; Saller and Bienz, 2001; Sivasankaran et al., 2000; Zhang et al., 2001), is suggested by evidence indicating that Dpp is required to override REPE function. In the wing and leg imaginal discs, *brk* expression is repressed by and is roughly reciprocal to Dpp signaling. Hence, in the leg disc, *brk* expression is lowest in dorsal-most leg cells (Jazwinska et al., 1999; Muller et al., 2003) (Fig. 6B). D-*h*-*GFP* is expressed within the region of low-level *brk* expression in leg discs (Fig. 6A). Furthermore, *brk* expression expands dorsally in $dpp^{d6}/dpp^{d12}$ legs (Fig. 6C), in which we showed that D-*h* expression is severely reduced (Fig. 5F).

To determine whether Brk functions as a repressor of D-*h* expression, we examined D-*h*-*GFP* expression in clones lacking *brk* function. Loss of *brk* function results in ectopic expression of D-*h*-*GFP* on either side of the D-*h*-GFP stripe. In Fig. 7A-C, ectopic expression is observed in clones anterior to the D-*h*-GFP stripe. However, the expansion is confined to a region two or three cells wide, directly juxtaposed to D-*h* expression, which presumably corresponds to the HHRE-responsive zone. In addition, we observe ectopic expression in ventral clones (Fig. 7D-F). We further observe that overexpression of *brk* along the AP boundary drastically reduces D-*h*-*GFP* expression but does not affect HHRE-*GFP* expression (Fig. 6D,E), indicating that Brk acts through the REPE to repress D-*h* expression. As D-*h* expression is activated primarily by the Hh-responsive HHRE, these observations identify Brk as repressor of Hh as well as Dpp target genes.

## A Brinker responsive repression element refines D-*h* expression

Genetic data support a hypothesis in which Brk acts through the REPE of the D-*h* enhancer to modulate activity of the HHRE. If so, we might expect the REPE to contain one or more functional Brk-binding sites. Hence, we searched the REPE for the Brk consensus binding site, GGCG(C/T)(C/T) (Kirkpatrick et al., 2001; Sivasankaran et al., 2000; Zhang et al., 2001), and identified a potential Brk binding site which overlaps two sequences similar to a consensus binding sites for Mad, GCCGNCGC (Kim et al., 1997), and a sequence similar to a cAMP response element (CRE), TGACGTCA (Montminy et al., 1986). The sequence of overlapping CRE, Brk and Mad sites was designated the CMB element (see Fig. 1B, Fig. 8A).

To determine whether Brk might act through the CMB element, we first tested whether Brk binds the CMB site in vitro. EMSA analysis shows that Brk binds the CMB-Brk site in a sequence-specific manner (Fig. 3, lanes 8-14). In addition,

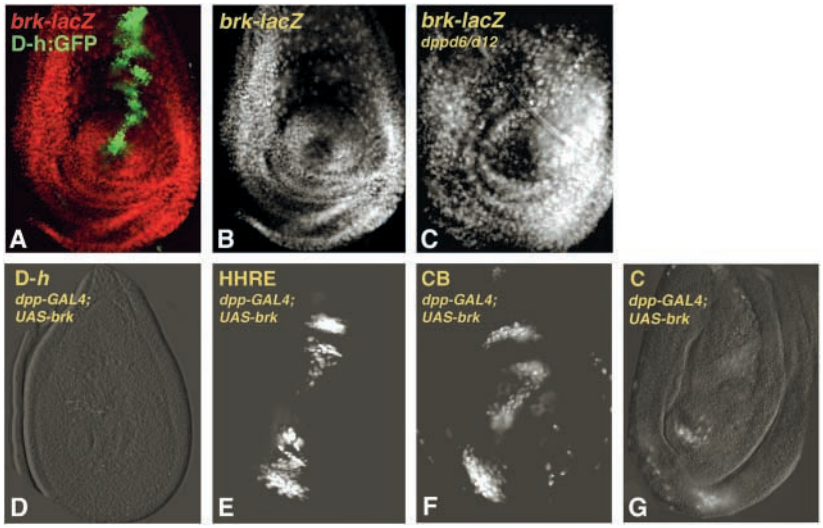



**Fig. 6.** Brk is a potential D-*h* repressor. (A) D-*h*-*GFP* expression (green) relative to *brk*-*lacZ* expression (red) in a wild-type leg disc. (B,C) *brk*-*lacZ* expression in a wild-type leg disc (B) and a dpp hypomorph (*dppd6/dppd12*) leg disc (C). (D,G) Overexpression of *brk*, in UAS-*brk*/+; *dpp*-*Gal4*/+ legs, results in drastic reduction of D-*h*-*GFP* expression (D) and D-*h*-C-*GFP* (G), while HHRE-*GFP* (E) and D-*h*-CB-*GFP* are still expressed (F). It appears that in legs overexpressing *brk*, there are fewer HHRE-GFP and D-*h*-CB-GFP-expressing cells. This is probably due to compromised growth of leg discs when Brk levels are elevated near the AP boundary (Jazwinska et al., 1999).

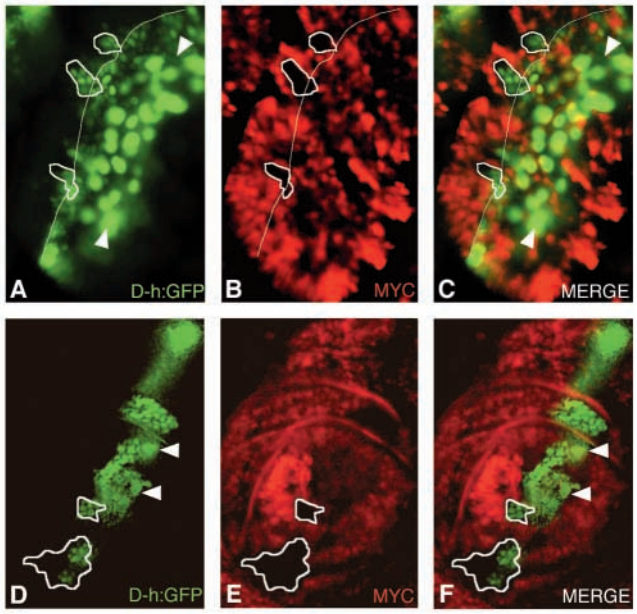



**Fig. 7.** Brk represses D-*h* expression. Clones lacking *brk* function were examined in a prepupal leg, 3 hours APF (A-C) and in a third instar leg disc (D-F). *brk*⁻ clones exhibit ectopic *GFP* expression anterior to the D-*h*-GFP stripe (A-C) and in the ventral leg (D-F). (A,D) D-*h*-*GFP* expression (small nuclei, green). Clones are outlined in white in all panels. White line marks the anterior boundary of D-*h*-*GFP* expression in A-C. Large *GFP*-expressing nuclei (arrowheads) probably correspond to adepithelial cells. (B,E) *myc* expression (red). *brk*⁻ clones are marked by the loss of *myc* expression. (C) Merge of images in A and B. (F) Merge of images in D and E.

we observed sequence-specific binding of Mad to the two potential Mad-binding sites in the CMB (Fig. 3, lanes 15-22). Next, the function of the Brk binding site was tested by mutational analysis. It was not possible to alter the Brk site without compromising at least one other site so the following sites were mutated within the CMB: Mad2 (D-*h*-M2) and Brk/Mad2 (D-*h*-BM2) (Fig. 8A). Brk and Mad binding to all Mad and CRE mutant versions of the CMB was confirmed by EMSA (Brk binds the M2 mutant probe but not the BM2 mutant probe) (Fig. 3, lanes 25, 26). Although D-*h*-M2-*GFP* expression appears normal (Fig. 8B), D-*h*-BM2-*GFP* expression is broader along the AP and DV axes (Fig. 8C). These observations are consistent with the hypotheses that Brk acts through the CMB to repress D-*h* expression.

As shown above, mutation of the Mad2 site has no effect on D-*h*-*GFP* expression, suggesting that only the Mad1 site is required, that the Mad2 site is functionally redundant with the Mad1 site, or that neither site is required for D-*h* expression. To test whether the Mad-binding sites are involved in D-*h* expression, both CMB-Mad sites were mutated (D-*h*-MM) (Fig. 8A). Weak residual binding to a single Mad site is observed with the CMB-MM mutant probe (not shown; see Materials and methods) and Brk binding is also reduced (Fig. 3, lane 27). We observe that D-*h*-MM-GFP stripe extends into the ventral disc and is slightly expanded along the AP axis (Fig. 8D, note that the stripe is two or three cells wide in the pupal leg). A probable explanation for this result is that ectopic expression is caused by compromised Brk binding to the MM-mutant CMB. We also observe that a D-*h*-MBM-*GFP* transgene directs expression of a stripe, which is broader along the AP and DV axes of the leg disc (Fig. 8E). The finding that the MBM mutation (which completely abrogates Brk binding) causes a more severe expansion phenotype than the MM mutation (which partially reduces Brk binding) is consistent with the hypothesis that the Brk binding site contributes to repression of D-*h* expression.

### A Brinker response site in the D-*h* repression element is not sufficient to mediate repression

We also asked whether the CRE is required for proper D-*h* expression. To test function of this site, a mutation was introduced into the CRE (D-*h*-C), such that Brk and Mad binding were not compromised (Brk binding to the CRE mutant is shown in Fig. 3, lanes 24). Surprisingly, mutation of the CRE results in expression very similar to that of the HHRE-*GFP* transgene. The D-*h*-C stripe extends ventrally and is five or six cells wide, which suggests that the CRE is required for D-*h* repression (Fig. 8G). Very similar expression is observed with D-*h*-CB- (Fig. 8F) and D-*h*-MCM-*GFP* (not shown)

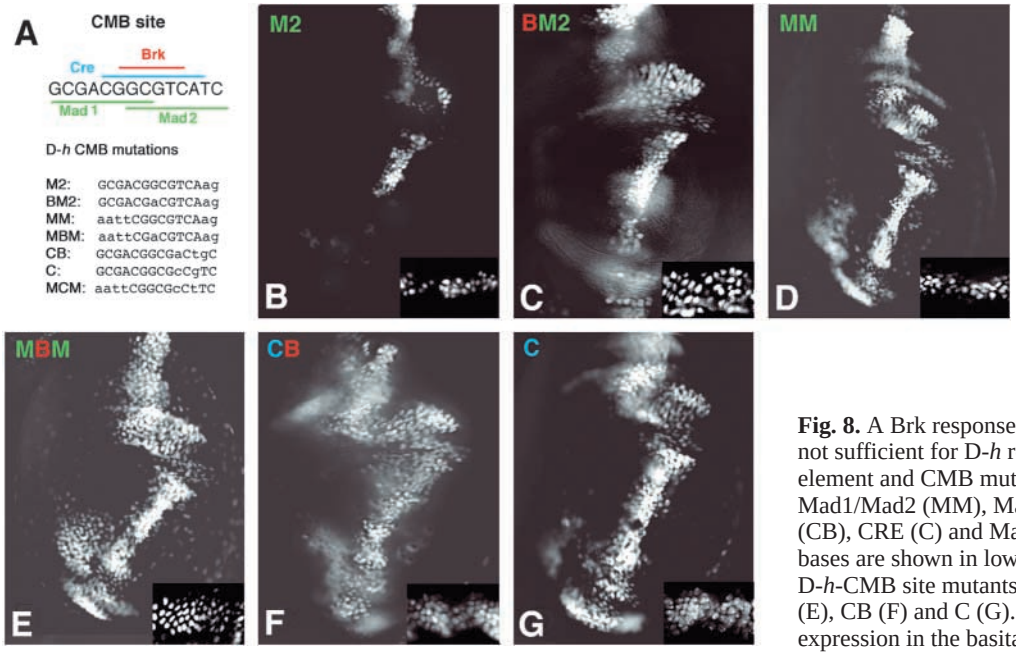



**Fig. 8.** A Brk response site in the D-*h* repression element is not sufficient for D-*h* repression. (A) Sequence of CMB element and CMB mutants: Mad2 (M2), Brk/Mad2 (BM2), Mad1/Mad2 (MM), Mad1/Brk/Mad2 (MBM), CRE/Brk (CB), CRE (C) and Mad1/CRE/Mad2 (MCM). Mutagenized bases are shown in lower case. (B-G) D-*h*-GFP directed by D-*h*-CMB site mutants: M2 (B), BM2 (C), MM (D), MBM (E), CB (F) and C (G). The insets in all panels show *GFP* expression in the basitarsal segment of a 4-hour prepupal leg.

transgenes. Although, it appears that Brk binds the CMB to mediate repression of HHRE-directed expression, it is evident that an intact Brk binding site is not sufficient for D-*h* repression.

Because of the extensive overlap of Mad, CRE and Brk sites in the CMB, it was necessary to alter one base pair in the CRE when the BM2 and MBM mutations were generated. Although this mutation alters the site so that it more closely resembles a canonical CRE (Fig. 8A), it is possible that it disrupts function of the CMB-CRE. This raises the possibility that Brk acts indirectly through the CRE rather than by directly binding the Brk site. To test this hypothesis, we assayed the effect of Brk overexpression on D-*h*-C-*GFP* and D-*h*-CB-*GFP* expression. Brk overexpresssion only mildly affects D-*h*-CB-*GFP* (Fig. 6F) but severely reduces D-*h*-C-*GFP* (Fig. 6G) expression, suggesting that Brk can act directly through its binding site in the CMB.

## Discussion

As a critical regulator of periodic *ac/sc* expression in the *Drosophila* leg disc, *h* performs a pivotal function in formation of adult leg morphology. We have previously shown that compromised function of the enhancers that control *h* expression in leg imaginal discs results in disruption of the adult leg microchaete pattern. Furthermore, in this and a previous study, we observed that the *h* leg stripe enhancers respond to distinct combinations and levels of the Hh, Dpp and Wg morphogens. Hence, *h* acts to link function of the morphogens that globally pattern leg imaginal discs to *ac/sc*, which act at a local level to define the primordia of the leg microchaete bristle rows. In order to gain insight into the molecular mechanisms underlying signal integration by the *h* gene, we undertook an analysis of the D-*h* enhancer. Dissection of the D-*h* enhancer led to the identification of activation and repression elements, which together establish a spatially defined stripe of *h* expression in the dorsal leg.

### Activation of D-*h* expression

The D-*h* activation element, HHRE, has two consensus Ci-binding sites, which bind Ci in vitro, and are required for its activity. In addition, HHRE-*GFP* expression is abrogated in clones lacking function of *smo*, a transducer of the Hh signal (Alcedo et al., 1996; van den Heuvel and Ingham, 1996). These observations suggest that Ci acts directly through the HHRE to activate D-*h* expression. *h* is one of a number of genes, including *dpp*, *patched* (*ptc*), *knot* and *araucan/caup* (*ara/caup*), that have been identified as targets of Hh signaling in imaginal discs (Aza-Blanc and Kornberg, 1999; Vervoort, 2000). These genes are each expressed in a stripe along the AP compartment boundary, but curiously, stripe widths among the genes varies as does register relative to the AP boundary. This has been explained in terms of differential response of Hh-target genes to the repressor and activator forms of Ci (Ci-R and Ci-A, respectively) found in anterior compartment cells (Methot and Basler, 1999; Muller and Basler, 2000; Wang and Holmgren, 1999). *ptc*, for example, has been proposed to respond only to the maximal levels of Ci-A found in cells nearest the AP boundary, while *dpp* responds to lower levels of Ci-A and also to Ci-R. The broad AP boundary stripe of HHRE-directed expression suggests that the HHRE is highly responsive to Ci-A. Differential response to Ci-R and Ci-A is thought to be controlled by cis-regulatory elements outside the local context (within 100 bp) of Ci binding sites in Hh responsive enhancers (Muller and Basler, 2000). Consistent with this hypothesis, we have identified an element, the REPE, which appears to modulate the response of the HHRE to Ci-A.

Although Ci-A is an essential and important activator, which acts directly through the HHRE, it is unlikely that Ci-A function is sufficient for HHRE activity. Several studies have suggested that signal response elements in enhancers are generally not sufficient to activate gene expression (Barolo and Posakony, 2002). Rather, the transcriptional effectors of signals must act cooperatively with other activators to direct robust expression of target genes. This phenomenon, which has been

termed 'activator insufficiency' (Barolo and Posakony, 2002), presumably prevents promiscuous activation of potential target genes. It is likely then, that other sites in the HHRE are required in addition to the Ci sites for expression directed by this element. For example, as the HHRE drives reporter gene expression in the wing and antennal discs (not shown) as well as the leg, we might expect a common factor expressed in all three discs to act through the HHRE in combination with Ci. Alternatively, the enhancer might harbor sites that respond to factors specific to each disc type.

### Dpp signals through the D-*h* repression element to block Brk-mediated repression

We have identified a short sequence in the REPE, the CMB, which functions to restrict HHRE expression to a narrow dorsal domain. In this study, we provide strong evidence for the hypothesis that the transcriptional repressor Brk acts through the CMB to repress D-*h* expression. Although previous studies have shown that *brk* expression is very low or undetectable in cells near the Dpp source, we observe a genetic requirement for *brk* in repression of D-*h* in this region. In addition, overexpression of *brk* results in a dramatic reduction of D-*h*-*GFP* expression, but only mildly affects expression from a D-*h*-*GFP* transgene with a compromised Brk binding site.

We also found that Dpp acts through the REPE to block Brk-mediated repression. We propose that high-level Dpp signaling defines the domain of D-*h* expression within the HHRE-response zone. This idea is supported by the observations that D-*h*-*GFP* but not HHRE-*GFP* expression is dependent on Dpp, indicating that Dpp signals through the REPE, and that elevation of Dpp signaling results in expansion of D-*h* expression along the AP and DV axes, within the domain of HHRE activity. Our current studies suggest that the function of Dpp in regulation of D-*h* expression may be limited to repression of *brk*. Yet, the presence of Mad-binding sites in the CMB suggests a potentially more direct role for activated Mad (act-Mad), the transcriptional mediator of Dpp signaling. Brk has been shown to be a potent competitor of Mad in vitro for binding to overlapping binding sites in Dpp target enhancers (Saller and Bienz, 2001). Hence, a potential role for Mad would be to prevent Brk from binding the CMB, thereby blocking Brk repression in cells receiving high-level Dpp signaling. If this model is correct, we might have expected the MM mutation to compromise D-*h* expression, which was not the case. However, the destabilization of Brk binding to the MM mutant might have masked a requirement for the Mad sites in blocking Brk repression.

It has recently been shown that an act-Mad/Shn complex represses *brk* expression by binding a silencer element (Muller et al., 2003). Therefore, as mutation of the Mad sites expands D-*h* expression, it is possible that Mad acts in concert with Brk through the CMB to repress D-*h* expression. This notion is not inconsistent with genetic evidence, indicating a requirement for *Mad* in D-*h* expression, as loss of *Mad* function elevates Brk levels, which (as discussed below) can overcome the requirement for CMB-sequences other than the Brk site. However, if this were the case, we might have expected a more severe expansion phenotype with the MM mutant, in which both Brk and Mad binding are compromised. Further analysis is required to determine the role, if any, of the CMB-Mad-binding sites in D-*h* expression.

### A CRE in the D-*h* repression element is required for repression

Given the genetic evidence that Brk represses D-*h* expression and that Brk binds the CMB element in vitro, the most straightforward hypothesis is that Brk acts directly through the CMB in vivo to repress D-*h* expression. However, as mutation of the CRE also causes loss of repression, it is formally possible that the CRE rather than the Brk site is important for repression. A potential explanation for this observation is that mutation of the CRE lowers the affinity of this element for binding to Brk, even though the Brk binding site is intact in the CMB-C mutant. Because the levels of Brk in the dorsal leg are limiting, altered affinity could have a significant effect on the level of Brk occupancy of the CMB. However, we observe through EMSA analysis that the CRE mutant CMB binds Brk with an affinity greater than that of the wild-type element (Fig. 3, lanes 23,24).

As it was not possible to mutate the Brk site without affecting the CRE, the CRE was altered in the BM2 and MBM mutants such that it more closely resembles a canonical CRE. Nevertheless, this change in the CRE may have affected its function. If so, this would be consistent with a model in which the CRE mediates repression of D-*h* expression, and Brk acts indirectly through the CRE rather than the Brk site. However, the finding that Brk overexpression drastically reduces D-*h*-C-*GFP* but not D-*h*-CB-*GFP* expression suggests that Brk can act directly through the Brk site, independent of the CRE.

The requirement for CMB-sequences outside the Brk binding site suggests that the context of the Brk site within the CMB is important for repression. A plausible explanation for the requirement of the CRE is that it is bound by a factor, X, which functions to facilitate recruitment of Brk under conditions where Brk levels are limiting, such as in the dorsal leg. Consistent with this hypothesis is the observation that overexpression of Brk greatly reduces D-*h*-C-*GFP* expression, suggesting that the requirement for the CRE can be bypassed if the levels of Brk are high enough. However, when Brk levels are limiting, the CRE might contribute more to D-*h* repression than the Brk site. For example, in the dorsal leg, Factor X might bind the CMB and then form a complex with Brk, relieving the necessity for Brk to bind the CMB directly. This model could explain why D-*h* expression appears to be significantly more sensitive to Brk-mediated repression than other Brk targets in imaginal discs, such as *vestigial* (*vg*) and *opotomotor-blind* (*omb*). *vg* and *omb* are each expressed in broad domains across the center of the wing disc and are repressed by higher levels of Brk than is D-*h* (Campbell and Tomlinson, 1999; Jazwinska et al., 1999; Minami et al., 1999). Perhaps, the CRE and/or other sequences in the REPE mediate heightened response to Brk. It will be of interest to determine whether other Brk-target genes, such as *spalt*, which are also repressed by very low levels of Brk, are similarly regulated.

A second potential function for a CRE-binding factor X is to act in concert with Brk to mediate D-*h* repression. Several lines of evidence suggest that Brk is a versatile repressor, which can inhibit transcription by competing with activators for binding to a common site or by active repression. Active repressors can act either at short range, by inhibiting activity of activators bound to nearby elements (150 bp away or less), or at long range by interfering with activators bound at a greater distances (Cai et al., 1996). Brk can mediate active repression

(Kirkpatrick et al., 2001), and binds the co-repressors dCtBP and Groucho (Gro), which mediate short- and long-range repression, respectively (Saller et al., 2002; Zhang et al., 2001). Brk requires Gro and/or dCtBP function for repression of a subset of its target genes, whereas neither is required for repression of others (Hasson et al., 2001). In the D-*h* enhancer, the CMB is positioned about 1 kb from the HHRE, suggesting that CMB-binding repressor(s) act at long range to repress HHRE-directed expression. Although Brk directly binds Gro, factor X could facilitate recruitment of Gro or other co-factors required for long-range repression.

### Integration of Dpp and other signals

In this study, we identify a novel function for Brk as repressor of Hh-target gene expression. Brk was originally identified as a repressor of Dpp-target genes (Campbell and Tomlinson, 1999; Jazwinska et al., 1999; Minami et al., 1999) and a recent study indicates that Brk can block Wg-mediated transcription as well (Saller et al., 2002). Brk was shown to antagonize function of a Wg-responsive element in the midgut enhancer of the Ultrabithorax (Ubx). The Ubx midgut enhancer drives Ubx expression in parasegment (ps) 7 of the embryonic midgut (Thuringer et al., 1993). Two elements, one which is Wg responsive (the WRS) and another Dpp responsive (the DRS) function synergistically to activate Ubx expression in ps 7 expression (Yu et al., 1998). In the adjacent ps8, however, Brk binds to the DRS and blocks the activity of the WRS (Saller et al., 2002). Curiously, the D-*h*-CMB and the Ubx-DRS are similarly organized in that each consists of overlapping CRE/Mad and Brk sites. The Ubx-DRS appears to mediate two modes of signal integration which involve: (1) synergistic activation, in which Mad/Med and dTCF act together to activate expression; and (2) activation and refinement, in which there is Wg mediated activation combined with Brk repression, which is blocked by Dpp. In the D-*h* enhancer, however, the CMB appears to be a component of a dedicated repression element, which appears to mediate only the second mode of signal integration: activation and refinement. The similar organization of the CMB and DRS suggests that it may be possible to predict the structure of enhancers known to be Brk responsive and which integrate Dpp and a second signal.

Despite the similarities, there are important distinctions between the D-*h* and Ubx-midgut enhancers, suggesting that the mechanisms of Brk-mediated repression might differ in each case. In the Ubx-midgut enhancer, the DRS and WRS are separated by 10 bp, suggesting that Brk acts at short range to inhibit WRS activity. In the D-*h* enhancer, however, the CMB is positioned at least 1 kb from the HHRE, implying a long-range effect for this element. Furthermore, Brk repression of the WRS depends on Teashirt (Tsh), which binds Brk and acts as a co-repressor (Saller et al., 2002). Tsh is unlikely to be required for D-*h* repression because it is only expressed in proximal leg segments (Erkner et al., 1999). Our studies suggest the requirement for a second DNA-bound factor, which binds the CRE, in addition to Brk for repression. The DRS-CRE, however, is required in addition to the Mad-binding sites for activation of Ubx in ps 7 (Eresh et al., 1997; Szuts et al., 1998).

### Competing inputs by Ci and Brk define a stripe of *hairy* expression

Together, our observations are consistent with a model (Fig. 9) in which Ci, acting through the HHRE, activates D-*h* expression. The domain of HHRE activity can be divided into two zones, 1 and 2 (Fig. 9). The HHRE has the potential to direct expression in both zones 1 and 2, but its activity is restricted to zone 1 by Brk and perhaps a second factor, X, which binds the CRE. In zone 2 cells, Brk would bind to the CMB and repress HHRE-directed expression. We propose that zone 1 is defined by the overlap of Hh and high-level Dpp signaling. Dpp promotes D-*h* expression by repressing *brk* expression in zone 1. However, the presence of Mad-binding sites in the CMB suggests the potential for a more direct role for Mad in D-*h* regulation, perhaps in competing with Brk for binding to the CMB, as shown, or in directly mediating repression. Confirmation of a role for the Mad sites awaits further analysis of the D-*h* enhancer.

### Connecting enhancer function to morphology

We show here that establishment of D-*h* expression in a defined domain is a complex process. This may be explained in part by the observation that morphological elements such as leg bristle rows are remarkably invariant in position from one individual to the next in *Drosophila melanogaster*. Hence, precise expression of genes such as *h*, the function of which is so crucial for positioning of elements such as the leg sensory bristles,

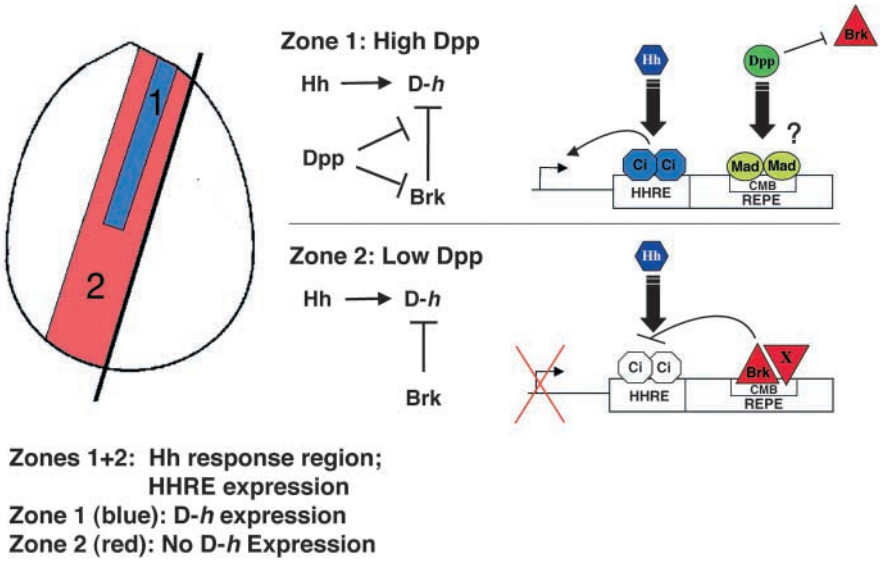



**Fig. 9.** Model for D-*h* regulation. Ci acts through the HHRE to activate expression in a broad AP boundary stripe that can be divided into zones 1 (blue) and 2 (red). The intact D-*h* element directs expression only in zone 1. Brk and perhaps a second factor, X (see text), act through the CMB to repress D-*h* expression in zone 2. In zone 1, Dpp signaling prevents Brk repression of D-*h* expression. Dpp function might be restricted to repression of *brk* expression in zone 1. However, it also possible that Mad acts more directly through the Mad-binding sites.

is essential. The organization of the D-*h* enhancer is reminiscent of that observed in another recently described enhancer, which is necessary for the development of a specific morphological element of an adult appendage. The *knirps* (*kni*) second longitudinal wing vein (L2) enhancer drives expression of *kni*, which is required to initiate L2 development, in a narrow stripe within the L2 primordia (Lunde et al., 2003). As observed with D-*h*, localized expression of *kn* in the L2 primordia is established by an enhancer consisting of discrete activation and repression elements. The activation element directs broad expression, which is refined by the repression element. The structure of the *kn* repression element appears complex in that it is thought to bind a number of repressors, including perhaps Brk. Although, we show here that the CMB element is important and essential for D-*h* repression, it is likely that there is a greater degree of complexity in the D-*h*-REPE, as well. Further analysis of the HHRE and REPE should provide mechanistic insight into how activation and repression elements function coordinately to establish precisely defined gene expression.

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## References

**Alcedo, J., Ayzenzon, M., Von Ohlen, T., Noll, M. and Hooper, J. E.** (1996). The Drosophila smoothened gene encodes a seven-pass membrane protein, a putative receptor for the hedgehog signal. *Cell* **86**, 221-232.

**Alexandre, C., Jacinto, A. and Ingham, P. W.** (1996). Transcriptional activation of hedgehog target genes in Drosophila is mediated directly by the cubitus interruptus protein, a member of the GLI family of zinc finger DNA-binding proteins. *Genes Dev.* **10**, 2003-2013.

**Aza-Blanc, P. and Kornberg, T. B.** (1999). Ci: a complex transducer of the hedgehog signal. *Trends Genet.* **15**, 458-462.

**Aza-Blanc, P., Ramirez-Weber, F. A., Laget, M. P., Schwartz, C. and Kornberg, T. B.** (1997). Proteolysis that is inhibited by hedgehog targets Cubitus interruptus protein to the nucleus and converts it to a repressor. *Cell* **89**, 1043-1053.

**Barolo, S. and Posakony, J. W.** (2002). Three habits of highly effective signaling pathways: principles of transcriptional control by developmental cell signaling. *Genes Dev.* **16**, 1167-1181.

**Barolo, S., Carver, L. A. and Posakony, J. W.** (2000). GFP and beta-galactosidase transformation vectors for promoter/enhancer analysis in Drosophila. *Biotechniques* **29**, 726, 728, 730, 732.

**Blair, S. S.** (1992). Engrailed expression in the anterior lineage compartment of the developing wing blade of Drosophila. *Development* **115**, 21-33.

**Blair, S. S. and Ralston, A.** (1997). Smoothened-mediated Hedgehog signalling is required for the maintenance of the anterior-posterior lineage restriction in the developing wing of Drosophila. *Development* **124**, 4053-4063.

**Brook, W. J. and Cohen, S. M.** (1996). Antagonistic interactions between wingless and decapentaplegic responsible for dorsal-ventral pattern in the Drosophila Leg. *Science* **273**, 1373-1377.

**Cai, H. N., Arnosti, D. N. and Levine, M.** (1996). Long-range repression in the Drosophila embryo. *Proc. Natl. Acad. Sci. USA* **93**, 9309-9314.

**Calleja, M., Renaud, O., Usui, K., Pistillo, D., Morata, G. and Simpson, P.** (2002). How to pattern an epithelium: lessons from achaete-scute regulation on the notum of Drosophila. *Gene* **292**, 1-12.

**Campbell, G. and Tomlinson, A.** (1999). Transducing the Dpp morphogen gradient in the wing of Drosophila: regulation of Dpp targets by brinker. *Cell* **96**, 553-562.

**Carroll, S. B. and Whyte, J. S.** (1989). The role of the hairy gene during Drosophila morphogenesis: stripes in imaginal discs. *Genes Dev.* **3**, 905-916.

**Chen, C. H., von Kessler, D. P., Park, W., Wang, B., Ma, Y. and Beachy, P. A.** (1999). Nuclear trafficking of Cubitus interruptus in the transcriptional regulation of Hedgehog target gene expression. *Cell* **98**, 305-316.

**Cubas, P., de Celis, J. F., Campuzano, S. and Modolell, J.** (1991). Proneural clusters of achaete-scute expression and the generation of sensory organs in the Drosophila imaginal wing disc. *Genes Dev.* **5**, 996-1008.

**Diaz-Benjumea, F. J., Cohen, B. and Cohen, S. M.** (1994). Cell interaction between compartments establishes the proximal-distal axis of Drosophila legs. *Nature* **372**, 175-179.

**Dominguez, M., Brunner, M., Hafen, E. and Basler, K.** (1996). Sending and receiving the hedgehog signal: control by the Drosophila Gli protein Cubitus interruptus. *Science* **272**, 1621-1625.

**Eresh, S., Riese, J., Jackson, D. B., Bohmann, D. and Bienz, M.** (1997). A CREB-binding site as a target for decapentaplegic signalling during Drosophila endoderm induction. *EMBO J.* **16**, 2014-2022.

**Erkner, A., Gallet, A., Angelats, C., Fasano, L. and Kerridge, S.** (1999). The role of Teashirt in proximal leg development in Drosophila: ectopic Teashirt expression reveals different cell behaviours in ventral and dorsal domains. *Dev. Biol.* **215**, 221-232.

**Gurdon, J. B. and Bourillot, P. Y.** (2001). Morphogen gradient interpretation. *Nature* **413**, 797-803.

**Hasson, P., Muller, B., Basler, K. and Paroush, Z.** (2001). Brinker requires two corepressors for maximal and versatile repression in Dpp signalling. *EMBO J.* **20**, 5725-5736.

**Hays, R., Buchanan, K. T., Neff, C. and Orenic, T. V.** (1999). Patterning of Drosophila leg sensory organs through combinatorial signaling by hedgehog, decapentaplegic and wingless. *Development* **126**, 2891-2899.

**Hepker, J., Blackman, R. K. and Holmgren, R.** (1999). Cubitus interruptus is necessary but not sufficient for direct activation of a wing-specific decapentaplegic enhancer. *Development* **126**, 3669-3677.

**Hepker, J., Wang, Q. T., Motzny, C. K., Holmgren, R. and Orenic, T. V.** (1997). Drosophila cubitus interruptus forms a negative feedback loop with patched and regulates expression of Hedgehog target genes. *Development* **124**, 549-558.

**Inaki, M., Kojima, T., Ueda, R. and Saigo, K.** (2002). Requirements of high levels of Hedgehog signaling activity for medial-region cell fate determination in Drosophila legs: identification of pxb, a putative Hedgehog signaling attenuator gene repressed along the anterior-posterior compartment boundary. *Mech. Dev.* **116**, 3-18.

**Jazwinska, A., Kirov, N., Wieschaus, E., Roth, S. and Rushlow, C.** (1999). The Drosophila gene brinker reveals a novel mechanism of Dpp target gene regulation. *Cell* **96**, 563-573.

**Jiang, J. and Struhl, G.** (1996). Complementary and mutually exclusive activities of decapentaplegic and wingless organize axial patterning during Drosophila leg development. *Cell* **86**, 401-409.

**Johnston, L. A. and Schubiger, G.** (1996). Ectopic expression of wingless in imaginal discs interferes with decapentaplegic expression and alters cell determination. *Development* **122**, 3519-3529.

**Kim, J., Johnson, K., Chen, H. J., Carroll, S. and Laughon, A.** (1997). Drosophila Mad binds to DNA and directly mediates activation of vestigial by Decapentaplegic. *Nature* **388**, 304-358.

**Kirkpatrick, H., Johnson, K. and Laughon, A.** (2001). Repression of dpp targets by binding of brinker to mad sites. *J. Biol. Chem.* **276**, 18216-18222.

**Lecuit, T., Brook, W. J., Ng, M., Calleja, M., Sun, H. and Cohen, S. M.** (1996). Two distinct mechanisms for long-range patterning by Decapentaplegic in the Drosophila wing. *Nature* **381**, 387-393.

**Lunde, K., Trimble, J. L., Guichard, A., Guss, K. A., Nauber, U. and Bier, E.** (2003). Activation of the knirps locus links patterning to morphogenesis of the second wing vein in Drosophila. *Development* **130**, 235-248.

**Marty, T., Muller, B., Basler, K. and Affolter, M.** (2000). Schnurri mediates Dpp-dependent repression of brinker transcription. *Nat. Cell Biol.* **2**, 745-749.

**Methot, N. and Basler, K.** (1999). Hedgehog controls limb development by regulating the activities of distinct transcriptional activator and repressor forms of Cubitus interruptus. *Cell* **96**, 819-831.

**Methot, N. and Basler, K.** (2000). Suppressor of fused opposes hedgehog signal transduction by impeding nuclear accumulation of the activator form of Cubitus interruptus. *Development* **127**, 4001-4010.

**Minami, M., Kinoshita, N., Kamoshida, Y., Tanimoto, H. and Tabata, T.**

(1999). brinker is a target of Dpp in Drosophila that negatively regulates Dpp-dependent genes. *Nature* **398**, 242-246.

**Modolell, J.** (1997). Patterning of the adult peripheral nervous system of Drosophila. *Perspect. Dev. Neurobiol.* **4**, 285-296.

**Montminy, M. R., Sevarino, K. A., Wagner, J. A., Mandel, G. and Goodman, R. H.** (1986). Identification of a cyclic-AMP-responsive element within the rat somatostatin gene. *Proc. Natl. Acad. Sci. USA* **83**, 6682-6686.

**Morimura, S., Maves, L., Chen, Y. and Hoffmann, F. M.** (1996). decapentaplegic overexpression affects Drosophila wing and leg imaginal disc development and wingless expression. *Dev. Biol.* **177**, 136-151.

**Muller, B. and Basler, K.** (2000). The repressor and activator forms of Cubitus interruptus control Hedgehog target genes through common generic gli-binding sites. *Development* **127**, 2999-3007.

**Muller, B., Hartmann, B., Pyrowolakis, G., Affolter, M. and Basler, K.** (2003). Conversion of an Extracellular Dpp/BMP Morphogen Gradient into an Inverse Transcriptional Gradient. *Cell* **113**, 221-233.

**Nellen, D., Burke, R., Struhl, G. and Basler, K.** (1996). Direct and long-range action of a DPP morphogen gradient. *Cell* **85**, 357-368.

**Nelson, H. B. and Laughon, A.** (1990). The DNA binding specificity of the Drosophila fushi tarazu protein: a possible role for DNA bending in homeodomain recognition. *New Biol.* **2**, 171-178.

**Ohsako, S., Hyer, J., Panganiban, G., Oliver, I. and Caudy, M.** (1994). Hairy function as a DNA-binding helix-loop-helix repressor of Drosophila sensory organ formation. *Genes Dev.* **8**, 2743-2755.

**Orenic, T. V., Held, L. I., Jr, Paddock, S. W. and Carroll, S. B.** (1993). The spatial organization of epidermal structures: hairy establishes the geometrical pattern of Drosophila leg bristles by delimiting the domains of achaete expression. *Development* **118**, 9-20.

**Patel, N. H., Martin-Blanco, E., Coleman, K. G., Poole, S. J., Ellis, M. C., Kornberg, T. B. and Goodman, C. S.** (1989). Expression of engrailed proteins in arthropods, annelids, and chordates. *Cell* **58**, 955-968.

**Penton, A. and Hoffmann, F. M.** (1996). Decapentaplegic restricts the domain of wingless during Drosophila limb patterning. *Nature* **382**, 162-164.

**Raftery, L. A. and Sutherland, D. J.** (1999). TGF-beta family signal transduction in Drosophila development: from Mad to Smads. *Dev. Biol.* **210**, 251-268.

**Romani, S., Campuzano, S., Macagno, E. R. and Modolell, J.** (1989). Expression of achaete and scute genes in Drosophila imaginal discs and their function in sensory organ development. *Genes Dev.* **3**, 997-1007.

**Rubin, G. M. and Spradling, A. C.** (1982). Genetic transformation of Drosophila with transposable element vectors. *Science* **218**, 348-353.

**Rushlow, C. A., Hogan, A., Pinchin, S. M., Howe, K. M., Lardelli, M. and Ish-Horowicz, D.** (1989). The Drosophila hairy protein acts in both segmentation and bristle patterning and shows homology to N-myc. *EMBO J.* **8**, 3095-3103.

**Rushlow, C., Colosimo, P. F., Lin, M. C., Xu, M. and Kirov, N.** (2001). Transcriptional regulation of the Drosophila gene zen by competing Smad and Brinker inputs. *Genes Dev.* **15**, 340-351.

**Saller, E. and Bienz, M.** (2001). Direct competition between Brinker and Drosophila Mad in Dpp target gene transcription. *EMBO Rep.* **2**, 298-305.

**Saller, E., Kelley, A. and Bienz, M.** (2002). The transcriptional repressor Brinker antagonizes Wingless signaling. *Genes Dev.* **16**, 1828-1838.

**Sivasankaran, R., Vigano, M. A., Muller, B., Affolter, M. and Basler, K.** (2000). Direct transcriptional control of the Dpp target omb by the DNA binding protein Brinker. *EMBO J.* **19**, 6162-6172.

**Skeath, J. B. and Carroll, S. B.** (1991). Regulation of achaete-scute gene expression and sensory organ pattern formation in the Drosophila wing. *Genes Dev.* **5**, 984-995.

**Spencer, F. A., Hoffmann, F. M. and Gelbart, W. M.** (1982). Decapentaplegic: a gene complex affecting morphogenesis in Drosophila melanogaster. *Cell* **28**, 451-461.

**Staehling-Hampton, K., Jackson, P. D., Clark, M. J., Brand, A. H. and Hoffmann, F. M.** (1994). Specificity of bone morphogenetic protein-related factors: cell fate and gene expression changes in Drosophila embryos induced by decapentaplegic but not 60A. *Cell Growth Differ.* **5**, 585-593.

**Szuts, D., Eresh, S. and Bienz, M.** (1998). Functional intertwining of Dpp and EGFR signaling during Drosophila endoderm induction. *Genes Dev.* **12**, 2022-2035.

**Tabata, T.** (2001). Genetics of morphogen gradients. *Nat. Rev. Genet.* **2**, 620-630.

**Theisen, H., Haerry, T. E., O'Connor, M. B. and Marsh, J. L.** (1996). Developmental territories created by mutual antagonism between Wingless and Decapentaplegic. *Development* **122**, 3939-3948.

**Thuringer, F., Cohen, S. M. and Bienz, M.** (1993). Dissection of an indirect autoregulatory response of a homeotic Drosophila gene. *EMBO J.* **12**, 2419-2430.

**Torres-Vazquez, J., Warrior, R. and Arora, K.** (2000). schnurri is required for dpp-dependent patterning of the Drosophila wing. *Dev. Biol.* **227**, 388-402.

**van den Heuvel, M. and Ingham, P. W.** (1996). smoothened encodes a receptor-like serpentine protein required for hedgehog signalling. *Nature* **382**, 547-551.

**Van Doren, M., Bailey, A. M., Esnayra, J., Ede, K. and Posakony, J. W.** (1994). Negative regulation of proneural gene activity: hairy is a direct transcriptional repressor of achaete. *Genes Dev.* **8**, 2729-2742.

**Vervoort, M.** (2000). hedgehog and wing development in Drosophila: a morphogen at work? *BioEssays* **22**, 460-468.

**Von Ohlen, T., Lessing, D., Nusse, R. and Hooper, J. E.** (1997). Hedgehog signaling regulates transcription through cubitus interruptus, a sequence-specific DNA binding protein. *Proc. Natl. Acad. Sci. USA* **94**, 2404-2409.

**Wang, Q. T. and Holmgren, R. A.** (1999). The subcellular localization and activity of Drosophila cubitus interruptus are regulated at multiple levels. *Development* **126**, 5097-5106.

**Wang, Q. T. and Holmgren, R. A.** (2000). Nuclear import of cubitus interruptus is regulated by hedgehog via a mechanism distinct from Ci stabilization and Ci activation. *Development* **127**, 3131-3139.

**Xu, T. and Rubin, G. M.** (1993). Analysis of genetic mosaics in developing and adult Drosophila tissues. *Development* **117**, 1223-1237.

**Yu, X., Riese, J., Eresh, S. and Bienz, M.** (1998). Transcriptional repression due to high levels of Wingless signalling. *EMBO J.* **17**, 7021-7032.

**Zarkower, D. and Hodgkin, J.** (1993). Zinc fingers in sex determination: only one of the two C. elegans Tra-1 proteins binds DNA in vitro. *Nucleic Acids Res.* **21**, 3691-3698.

**Zhang, H., Levine, M. and Ashe, H. L.** (2001). Brinker is a sequence-specific transcriptional repressor in the Drosophila embryo. *Genes Dev.* **15**, 261-266.

**Zimmerman, C. M. and Padgett, R. W.** (2000). Transforming growth factor beta signaling mediators and modulators. *Gene* **249**, 17-30.