Patterning of Drosophila Sense Organs

via Global Activation and Repression of

the Proneural Gene Achaete

BY

JI INN LEE
B.S., Kyung Hee University, Seoul, Korea, 2000
B.A., State University of New York at Buffalo, 2002
M.S., New York University, 2004

THESIS

Submitted as partial fulfillment of the requirements
for the degree of Doctor of Philosophy in Biological Sciences
in the Graduate College of The
University of Illinois at Chicago, 2012

Chicago, Illinois

Defense Committee:

Jennifer Schmidt, Chair
Ronald Dubreuil
Alisa Katzen, Biochemistry and Molecular Genetics
Peter Okkema
Teresa Orenic, Advisor

ACKNOWLEDGEMENTS

I would like to thank my advisor, Dr. Teresa Orenic, for her mentoring during my graduate school years. I am very grateful for her constant encouragement, support and advice. Also, aside from her passion for science, her profound knowledge and curiosity in a wide variety of subjects have been a great inspiration for me to further efforts.

I would also like to thank my committee members, Dr. Ronald Dubreuil, Dr. Alisa Katzen, Dr. Peter Okkema, and Dr. Jennifer Schmidt, for their insightful ideas and suggestions for my research and I am grateful for their generous time.

Many thanks go to the past and current Orenic Lab members for their support in the lab. I would like to especially thank Emily Wyskiel and Dr. Denis Bulanin. Emily has been a good friend and her pleasant personality has made the lab a fun place to be around. I am grateful for our friendship and her help in many ways. I thank Dr. Denis Bulanin for many helpful advices for the experiments and for helping me with microinjection.

I would like to thank Dr. Mella Rovani for our friendship and her kindness. I'm grateful for her helpful suggestions for experiments. I would like to thank Pastor Gray and friends at FPC.

I would like to thank my brother, Dr. Jiwoon Lee, who also studies developmental biology, for his encouragement and very helpful troubleshooting suggestions for experiments.

Lastly, but most importantly, I am immensely grateful to my parents for their love and faith, tremendous encouragement and prayers.

TABLE OF CONTENTS

CHAPTER PAGE

CHAPTER PAGE

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES (continued)

LIST OF FIGURES (continued)

LIST OF ABBREVIATIONS

A	Anterior
ac	*achaete*
ACS	Achaete Scute Complex
A/P	anterior/posterior
APF	after puparium formation
bHLH	basic helix loop helix
Ci	Cubitus interruptus
Ci-A	activator form of Ci
Ci-R	repressor form of Ci
CRE	*cis*-regulatory element
CS	chemosensory
CS mC	chemosensory microchaete
D	Dorsal
D-*h*	Dorsal *hairy*
Dl	Delta
dpp	*decapentaplegic*
D/V	dorsal/ventral
en	*engrailed*

LIST OF ABBREVIATIONS (continued)

E(spl)	Enhancer of split
GFP	green fluorescent protein
h	*hairy*
H	Hairy
Hh	Hedgehog
MS	mechanosensory
MS mC	mechanosensory microchaetae
MS MC	mechanosensory macrochaetae
N	Notch
NICD	Notch intracellular domain
P	Posterior
PNS	peripheral nervous system
P/D	proximal/distal
Ptc	patched
rn	*rotund*
Sc	Scute
Smo	Smoothened
SO	sensory organ
SOP	sensory organ precursor

LIST OF ABBREVIATIONS (continued)

Su(H)	Supressor of Hairless
T2	second thoracic segment
V	Ventral
V-*h*	Ventral *hairy*
Wg	Wingless

SUMMARY

During vertebrate and invertebrate development, organs and tissues must be precisely patterned, and periodic proneural gene expression is an early and essential event in this process. The *Drosophila melanogaster* sensory bristles are a good model system to study the molecular mechanisms involved in controlling precise proneural gene expression. There are two classes of sensory bristles: early specified (mechanosensory macrochaetae and chemosensory microchaetae) and late specified (mechanosensory microchaetae) bristles.

Previous studies suggest that patterning of early-specified bristles requires induction of proneural gene expression at specific locations in the limb and body wall primordia. Proneural gene expression in primordia of early-specified bristles is controlled by discrete modular *cis*-regulatory elements (CRE). Our studies, however, suggest that a different mechanism is used to pattern late specified bristles: expression of the proneural gene, *achaete* (*ac*), in primordia of late specified bristles is controlled by a single CRE.

On the surface of the *Drosophila* leg, small mechanosensory microchaetae (mC) are organized in a series of longitudinal rows along the leg circumference. In the prepupal leg, *ac* is expressed in longitudinal stripes, which comprise the leg microchaete primordia. We have found that Hairy (H) and Delta/Notch (Dl/N) signaling function concertedly and non-redundantly to define periodic *ac* expression. This process involves broad and late activation of *ac* expression and refinement in response to a prepattern of repression, which is established by Hairy and Delta. These findings have allowed us to formulate a general model for generation of periodic bristle patterns in the adult leg, and this model is supported by the

analysis of a CRE, called the *ac*-mC-CRE, that specifically directs *ac* expression in the leg and notal mC proneural fields. This CRE contains an activation element, which directs broad expression of *ac* along the circumference of prepupal legs, and a repression element, which is Hairy and Dl/N responsive.

To gain insight into the molecular mechanisms that mediate repression by Hairy and Notch, we undertook a detailed analysis of the *ac*-mC-CRE. Our findings provide novel insight into repression of proneural *ac* expression by bHLH repressor, Hairy, and Notch, which acts through the bHLH Enhancer of split E(spl) repressors. We find that the response sites for these bHLH repressors is less specific than previously suggested. For example, Hairy represses *ac* expression via two sequences that contain a C-box core, consistent with previous observations. However, we present strong evidence suggesting that Hairy can also act through an E(spl)-like sequence that has a divergent core sequence. Furthermore, Notch appears to signal through two Hairy binding sites in the *ac*-mC-CRE. Previous reports have suggested that the Hairy and E(spl) proteins act through distinct target sequences. We find, however, that Hairy and E(spl) can mediate repression of *ac* via common sequences in the *ac* promoter and *ac*-mC-CRE. This finding has interesting implications regarding the evolution of morphological novelty in mC patterning.

I. INTRODUCTION

During vertebrate and invertebrate development, components of the nervous system must be precisely patterned spatially and temporally and this is achieved by precise regulation of proneural and neurogenic genes. How this pattern is established during neurogenesis has been one of the most fundamental and interesting questions. The purpose of this research is to understand the mechanisms underlying the pattern formation of sensory organs which comprise the peripheral nervous system (PNS). The *Drosophila* leg is a good model system to study pattern formation of the PNS; *Drosophila* bears innervated sensory bristles which are precisely positioned in a stereotyped pattern around the leg circumference. There are two types of sensory bristles based on function; Mechanosensory (MS) bristles, macrochaetae (MC, large) and microchaetae (mC, small), and chemosensory microchaetae (CS mC). They can also be classified according to the specification time; early specified (mechanosensory macrochaetae and chemosensory microchaetae) and late specified (mechanosensory microchaetae) bristles. Previous studies in the dorsal mesothorax, or notum, suggest that *achaete* (*ac*) expression in the primordia of early specified bristles is induced in specific positions and controlled by discrete modular enhancers (Campuzano and Modolell, 1992; Gomez-Skarmeta et al., 2003). However, our genetic studies suggest that a different mechanism is used for patterning of late specified bristles; *ac* expression in the primordia of late specified bristles (mechanosensory microchaete) is controlled by a single enhancer. The genetic studies of our laboratory suggest that *ac* expression can be broadly activated by

unknown activators but spatially refined into the eight longitudinal leg mC proneural stripes by prepattern regulators, Hairy (H) and Delta/Notch (Dl/N) signaling.

The focus of this research is on understanding how this periodic *achaete* expression in mechanosensory microchaete proneural fields is regulated at the molecular level. The molecular and genetic studies have identified a single *cis*-regulatory element (CRE) that directs *ac* expression in all the mC proneural fields in the leg as well as in the notum. Consistent with the genetic data, this CRE is responsive to Hairy and Notch signaling. Since there are remarkably similar mechanisms and sets of genes that control neuronal pattern formation between vertebrate and invertebrate, this study can provide general insight into the mechanisms controlling pattern formation during vertebrate development.

1.1. Pattern formation of peripheral nervous system in *Drosophila melanogaster*

The adult fly peripheral nervous system (PNS) includes more than 6000 external sensory organs that are organized in stereotyped arrays of bristles covering most of the body surface. The patterning of these sensory organs is a progressive and highly regulated process involving several steps as shown in Figure 1 (Fisher and Caudy, 1998); initially, pattern of nervous system is established by spatially restricted expression of prepattern regulator genes. The prepattern regulators (Hairy and Delta/Notch signaling in fly legs, discussed in later sections) are expressed before neurogenesis to define the position of future sensory organs, which arise from a group of cells expressing proneural genes, *achaete* (*ac*) and *scute* (*sc*). Expression of these proneural genes confers the potential to adopt neural cell fates on small groups of cells, called proneural clusters (Cubas et al., 1991; Skeath and Carroll, 1991). One

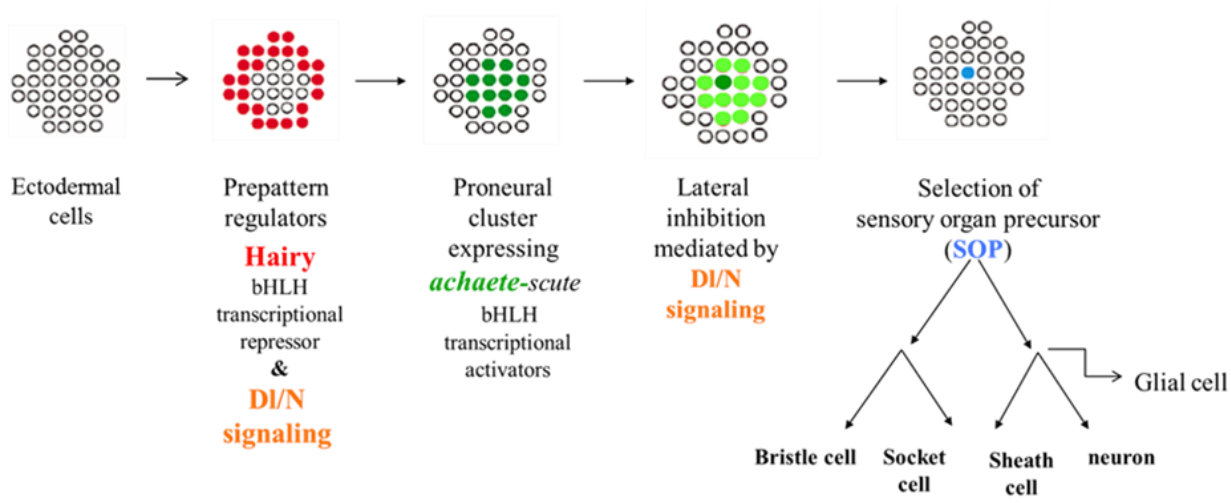


Figure 1. Overview of *Drosophila* sensory organ patterning

This figure is modified from Fisher and Caudy, 1998. In *Drosophila* imaginal discs, all ectodermal cells have the potential to acquire either an epidermal or neuronal identity. Initially, prepattern regulator genes (marked in red) are expressed in the ectodermal cells in response to global patterning regulators. Then, proneural genes (green) are expressed in groups of cells, called proneural fields. Proneural gene expression is spatially defined by the prepattern regulators. All cells in the proneural fields have the potential to become sensory organ precursors (SOP, blue), but only one or a few cells (dark green) are selected as SOPs, in part through a mechanism called lateral inhibition, mediated by Dl/N signaling. This selected SOP undergoes cell divisions and gives rise to the components of sensory bristles, bristle cell, socket cell, sheath cell, and neuron.

or a few cells in the proneural cluster, which displays the highest level of proneural protein Ac and Sc accumulation, are selected to become sensory organ precursor (SOP) and the rest of the cells adopt an epidermal cell fate. This process of singling out, called lateral inhibition, is mediated by the Delta (Dl)/Notch (N) signaling pathway (Hartenstein and Posakony, 1990; Simpson, 1990) . Then, the selected SOP undergoes a number of asymmetric cell divisions to give rise to four components of one sensory organ (Lai and Orgogozo, 2004; Posakony, 1994): SOP is divided by two distinct daughter cells, pIIa, and pIIb. pIIa produces two cells that form the external structures, bristle and its socket. pIIb is divided by glial cell, which undergoes apoptosis (Fichelson and Gho, 2003), and pIIIb, which produces the neuron and the sheath cell.

1.2. *Drosophila* leg development

1.2.1. Overview of Drosophila leg development

The *Drosophila* adult has three pairs of legs, one pair on each thoracic segment. Along the proximal to distal (PD) axis, each leg consists of nine segments, coxa, trochanter, femur, tibia, tarsus (five tarsal segments, the most proximal segment is called basitarsus). It also has pretarsus which bears two claws at the most distal end. In *Drosophila*, the adult leg develops from the leg imaginal disc, a sac-like structure composed of an epithelial monolayer derived from groups of embryonic ectodermal cells. Patterning of the leg imaginal disc is organized by a few conserved signaling molecules such as Hedgehog (Hh), Decapentaplegic (Dpp) and

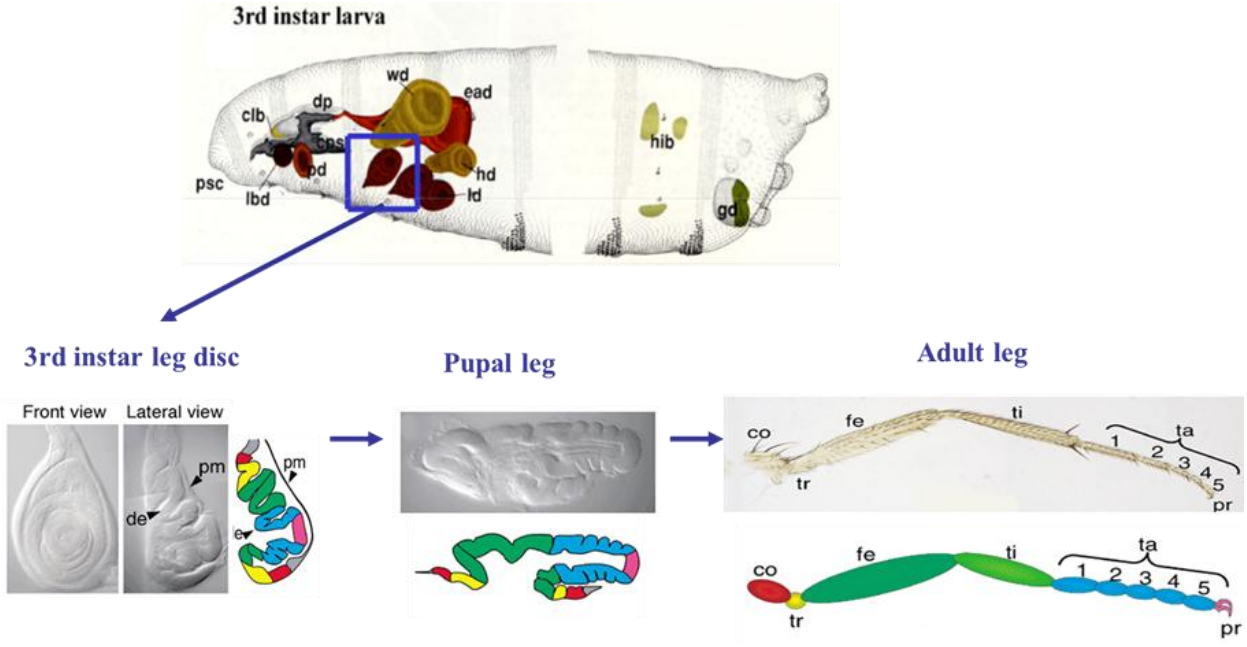


Figure 2. *Drosophila* leg development

This figure is adapted from Kojima, 2004 and Atlas of Drosophila Development by Hartenstein, 1993. In *Drosophila*, the primordia of adult limb structures are imaginal discs. The imaginal disc is a sac composed of an epithelial monolayer and named after the appendage it forms, such as the leg disc and the wing disc. During the 3rd larval instar, the leg disc is highly folded. During the pupal stage, the leg disc unfolds and telescopes out from the center, corresponding to the distal end, to acquire a cylindrical structure like the adult leg. The adult leg is composed of nine segments, from proximal to distal: coxa, trochanter, femur, tibia, and five tarsal segments.

Wingless (Wg) (Gurdon and Bourillot, 2001; Tabata, 2001). Each pathway is used repeatedly during the development of organisms, activating different subsets of target genes in different developmental contexts (Barolo and Posakony, 2002). During third instar larva development, the leg is highly folded. During pupal stage, the highly folded larval structure undergoes eversion and telescopes out to acquire cylindrical structure like adult structure as illustrated in Figure 2 (Kojima, 2004; Morata, 2001).

1.2.2. Patterning of *Drosophila* leg disc

The leg imaginal disc is divided into two populations of lineally distinct cells, which do not mingle, and they are called anterior (A) and posterior (P) compartments (Figure 3, (Basler and Struhl, 1994). In posterior compartment, cells express Hh, a short range secreted signaling molecule (Tabata and Kornberg, 1994). Hh diffuses to the anterior compartment and activates expression of its target genes near the Anterior-Posterior compartment boundary (A/P boundary) through its transcription mediator Cubitus interruptus (Ci), a zinc finger transcription factor (Alexandre et al., 1996). In the absence of Hh signaling, a transmembrane receptor Patched (Ptc), which is specifically expressed in anterior compartment, inhibits the function of another transmembrane receptor Smoothened (Smo) (Alcedo et al., 1996; Chen and Struhl, 1996; van den Heuvel and Ingham, 1996). Upon Hh signaling, Hh relieves Ptc repression on Smo ((Marigo and Tabin, 1996) and accumulates an activator form of Ci, a zinc finger transcription mediator of Hh pathway(Aza-Blanc et al., 1997). Ci then activates the Hh target genes, *dpp* dorsally and *wg* ventrally, along the A/P boundary (Dominguez et al., 1996; Hepker et al., 1997; Von Ohlen et al., 1997). Dpp is a

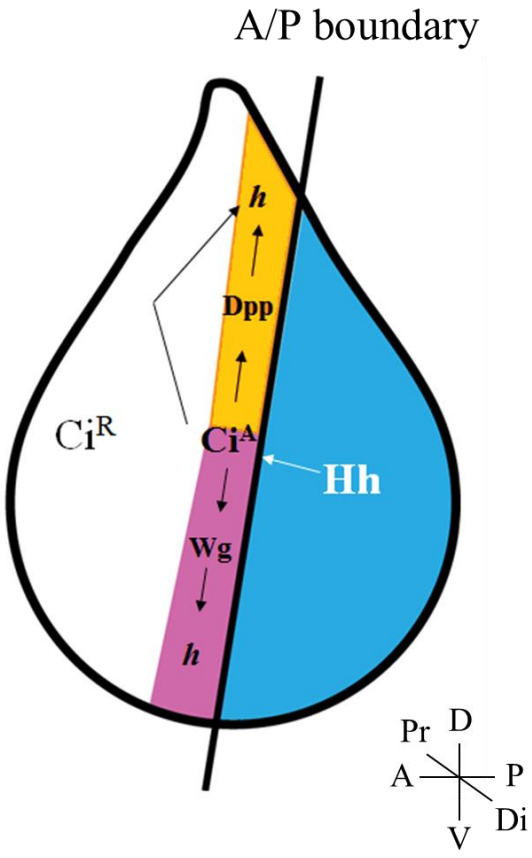


Figure 3. Patterning of *Drosophila* leg disc is mediated by conserved signaling molecules, Hh, Dpp and Wg.

The leg imaginal disc is divided into two lineally distinct compartments, the anterior (A) and posterior (P) compartments. Hh is a short range signaling molecule and is produced in the P compartment. It is secreted and diffuses into the A compartment, where it signals to cells near the A/P boundary, activating expression of its target genes. In response to Hh signaling, Ci, the transcriptional mediator of the Hh pathway accumulates in its activator form, Ci^A. Ci^A then activates expression of the Hh target genes, *dpp*, in a dorsal stripe near the A/P boundary and *wg* in a ventral A/P boundary adjacent stripe.

member of the transforming growth factor-beta (TGF-β) family of signaling molecules. Dpp acts as a long-range morphogen and specifies dorsal leg fate by regulating gene expression in a concentration-dependent manner (Lecuit et al., 1996; Nellen et al., 1996). Dpp is expressed near the A/P boundary and functions as an A/P organizer of the disc. Wg specifies a ventral leg fate and organizes the pattern of the ventral leg (Brook and Cohen, 1996; Jiang and Struhl, 1996; Penton and Hoffmann, 1996; Theisen et al., 1996). It has been shown that Dpp and Wg are mutually antagonistic in the patterning of the leg disc (Diaz-Benjumea et al., 1994; Lecuit and Cohen, 1997).

1.2.3. Drosophila sensory bristle development

Drosophila PNS consists of numerous sensory bristles on the body surface and these bristles are organized in stereotypical patterns. The specific patterns are established by precise expression patterns of proneural and prepattern genes, as mentioned previously. There are three different types of sensory bristles and they can be classified by function and specification time as shown in Figure 4 (Held and Heup, 1996): There are two types of sensory bristles based on function; Mechanosensory (MS) bristles, macrochaetae (MC, large) and microchaetae (mC, small), and chemosensory microchaetae (CS mC). They can also be classified according to the specification time; early specified (mechanosensory macrochaetae and chemosensory microchaetae) and late specified (mechanosensory microchaetae) bristles. The extensive studies on patterning of macrochaete, early specified bristle, in the notum and the recent data on patterning of microchaete, late specified bristles, have suggested that different molecular mechanisms are utilized in patterning two different types of sensory organs.

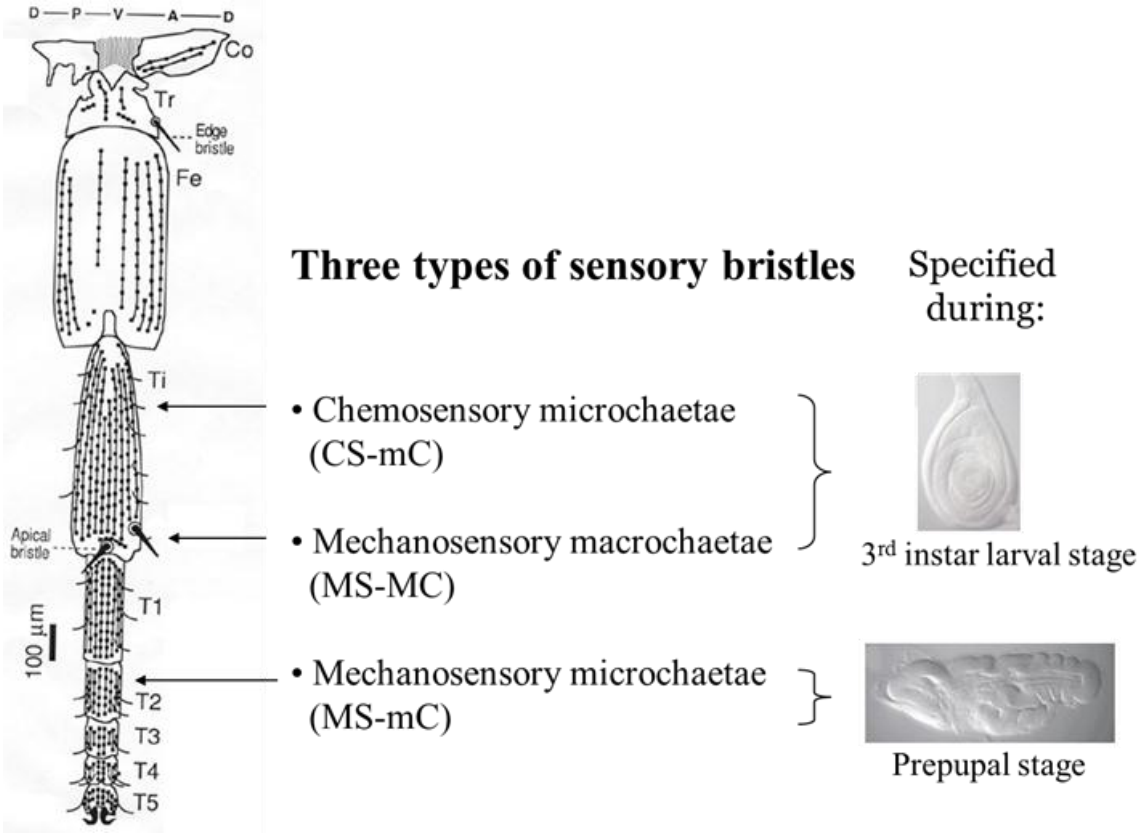


Figure 4. Various sensory bristle types are found on the *Drosophila* adult T2 leg.

This figure is adapted from Held and Heup, 1996 and Kojama, 2004. Schematic diagram of the adult T2 leg exhibits three different types of external sensory bristles found on the adult legs. These sensory organs can be grouped into two categories, based on the stage during which they are specified: the few chemosensory microchaetae (CS mC, small bristles) and few mechanosensory macrochaetae (MS MC, large bristlesare specified during the 3rd larval instar and early prepupal stages. The more numerous mechanosensory microchaetae (MS mC) are specified during mid-prepupal development.

The half of the notum, also called heminotum, is derived from the dorsal portion of the wing imaginal disc (Figure 5). On each *Drosophila* heminotum, there are eleven large bristles, or MC, at specific positions and about one hundred small bristles, or mC, are organized in longitudinal rows. These notal macrochaete are derived from sensory organ precursors, which are selected from a group of cells, called proneural cluster, expressing the redundant proneural genes *ac* and *sc*. In the MC primordia, proneural genes are expressed in the proneural clusters during the third instar larval stage but in the mC primordia, proneural genes are expressed during the prepupal stages, about 6 h APF.

The patterning of MC on the notum has been extensively studied (Calleja et al., 2002; Gomez-Skarmeta et al., 2003). It has been shown that, for MC proneural clusters expressing proneural genes *ac* and *sc*, there are several discrete *cis*-regulatory elements (CREs) scattered over the *ac-sc* locus (represented in colored boxes in Figure 5); Each element are shared by *ac* and *sc*, and responds to a specific combination of transcriptional activators and repressors that are expressed in the overlapping pattern in the notal disc, which therefore prepatterns the positions of future MCs. The best example is the patterning of dorsocentral (DC) macrochaete. Several prepattern factors including signaling molecules and transcription factors, alone or in combination, act through the DC enhancer to direct *ac/sc* expression in the DC macrochaete primordia. *ac/sc* expression in the proneural cluster for the DC bristles is directed by DC enhancer shown in pink in the Figure 5 during the 3rd instar stage (reviewed in Gomez-Skarmeta et al., 2003) and therefore, two SOPs are selected from that proneural cluster and give rise to the DC macrochaete on the notum.

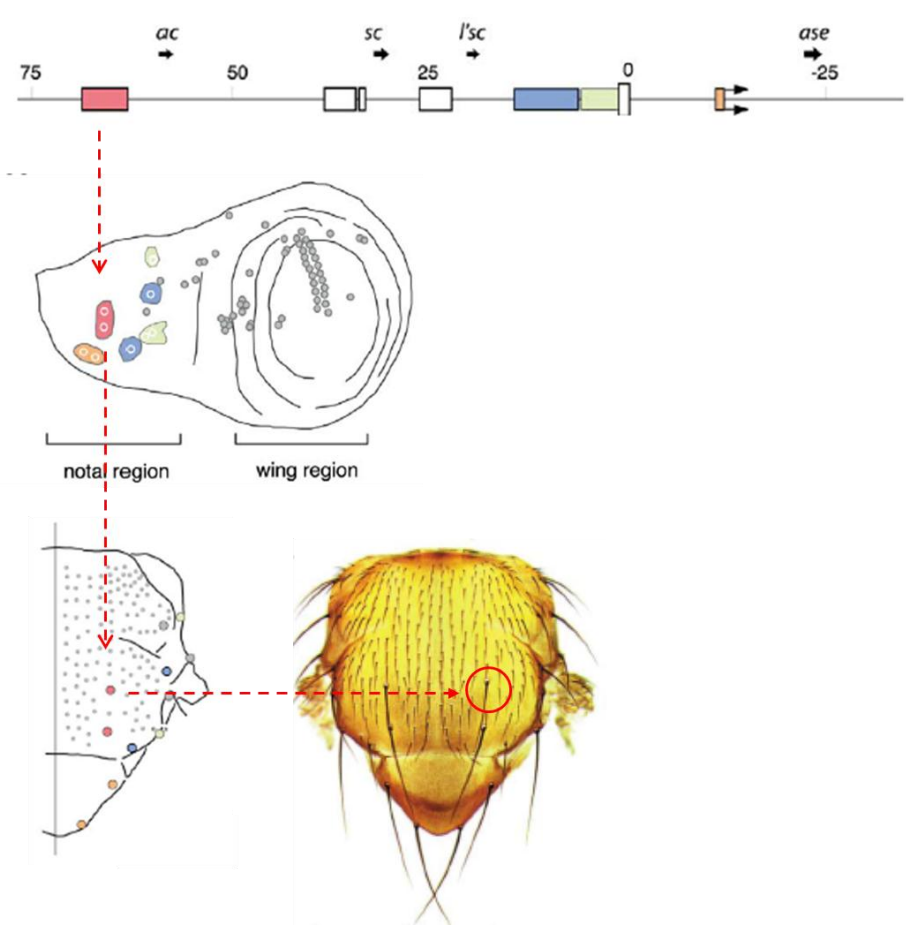


Figure 5. Schematic representation of macrochaete patterning in the *Drosophila* notum.

This figure is modified from Calleja et al, 2002. Several discrete *cis*-regulatory elements (CREs), represented by colored boxes, are distributed throughout the *ac/sc* complex. The left and right halves of the notum are each derived from the notal region of a wing imaginal disc. Each CRE regulates proneural gene expression in a proneural cluster of corresponding color, and therefore, macrochaetae development in the corresponding position.

1.3. Patterning of sensory organs in *Drosophila*

1.3.1. *achaete-scute* Complex in sensory organ formation

The *achaete-scute* complex (AS-C) contains four genes, *achaete (ac), scute (sc), lethal of scute (l'sc),* and *asense (ase),* that encode related basic-helix-loop-helix (bHLH) transcriptional regulators involved in various aspects of neurogenesis. Sensory organ precursor (SOP) development depends primarily on expression of the redundant genes, *ac* and *sc* (Cubas et al., 1991; Romani et al., 1989; Skeath and Carroll, 1991). *ac* and *sc* are expressed in clusters of cells located at precisely determined positions and one or a few SOPs are selected within these clusters of *ac-sc* expressing cells. Loss of function mutations in *ac* and *sc* results in the loss of defined subsets of neural progenitors and sensory organs (SOs) and ectopic expression of *ac* and *sc* promotes development of extra SOs in ectopic positions (Cubas et al., 1991; Skeath and Carroll, 1991; Romani et al, 1989). As mentioned, since AS-C proteins confer on cells the potential to become sensory organ precursors, these genes are called proneural genes and the clusters of cells expressing them are called proneural clusters (Ghysen and Dambly-Chaudiere, 1989; Romani et al., 1989). *l'sc* has an essential role in the generation of neuroblasts during development of the central nervous system (CNS) (Jimenez and Campos-Ortega, 1990) and *ase* functions for the proper differentiation of SOP (Dominguez and Campuzano, 1993; Jarman et al., 1993).

1.3.2. bHLH transcription factors in development of the nervous system

Transcriptional regulation by conserved bHLH transcription factors, activators and repressors, play key roles in many different developmental processes including neurogenesis in *Drosophila* and vertebrate organisms (Davis and Turner, 2001; Fisher and Caudy, 1998; Gaston and Jayaraman, 2003; Gray and Levine, 1996). The bHLH transcriptional factors function as either homo- or heterodimers and bind to common DNA sequences to activate and repress transcription of target genes (Murre et al., 1989). The basic regions of bHLH proteins make contact with the DNA, while the HLH domains are involved in dimerization. The common binding sites of bHLH factors are the sequences called E-box (CANNTG), C-box (CACNNG) and N-box (CANNTG) (Grove et al., 2009; Powell and Jarman, 2008). During development of the nervous system, the transcriptional activators have a proneural function and promote specification of a neural cell fate. The redundant proneural genes, *ac* and *sc,* are transcriptional activators and they specify neural cell fate in Drosophila. Daughterless (Da), a ubiquitously expressed bHLH protein, functions as a positive regulator of neurogenesis (Cabrera and Alonso, 1991). It has been shown that the proneural proteins Ac and Sc form heterodimers with Da and bind specifically to the E-box sequences (Cabrera et al., 1987; Murre et al., 1989).

There are also negative regulators that antagonize expression and/or function of bHLH proneural activators, including Hairy (H) / Enhancer of split [E(spl)] related proteins and Extramachrochaete (Emc). Hairy, a bHLH transcriptional repressor functions as a prepattern regulator of the *ac* proneural gene by directly binding to CACGCG, a core sequence of C-box, in the *ac* promoter in the wing (Ohsako et al., 1994; Van Doren et al., 1994). The E(spl) complex genes are the most widely expressed Notch signaling target, and seven of them

encode bHLH transcription factors and repress proneural gene expression (Davis and Turner, 2001). It has been shown that E(spl) proteins binds to a TGGCACGTG/CT/CA sequence, which includes a core E-box sequence, CACGTG (Jennings et al, 1999). The functions and regulation of Hairy and E(spl) are further discussed in the following sections. Another type of prepattern gene *emc* encodes a transcription factor of the HLH family that lacks the basic domain that is involved in the interaction with DNA (Van Doren et al., 1991). Emc heterodimerizes with proneural bHLH transcription factors Ac and Sc and sequesters them in inactive forms, thereby downregulating their proneural function (Campuzano, 2001).

1.3.3. Prepattern regulators in the *Drosophila* leg

The proneural gene *achaete* (*ac*) encodes a bHLH transcription factor and specifies neural cell fate on the Ac expressing clusters of cells in ectoderm. At 6 h APF, *ac* is expressed in a series of eight longitudinal stripes in the second leg (Figure 6B) and these longitudinal stripes of Ac expression mark the primordia of the mechanosensory microchaete (MS mC) bristle rows in the adults (Figure 6A). Ac expression in these stripes is necessary for formation of the MS mC bristle rows; if *ac* expression is lost or ectopically expressed (Figure 6D), it results in loss or ectopic formation of bristles (Figure 6C), respectively. The proper periodic expression of *ac* in eight longitudinal stripes in the prepupal leg is established by two prepattern regulators, Hairy and Delta/Notch signaling (Joshi et al., 2006; Orenic et al., 1993).

Figure 6. The *Drosophila* leg microchaetae and *ac* expression patterns in wild type and *hairy* mutant legs (Orenic et al., 1993).

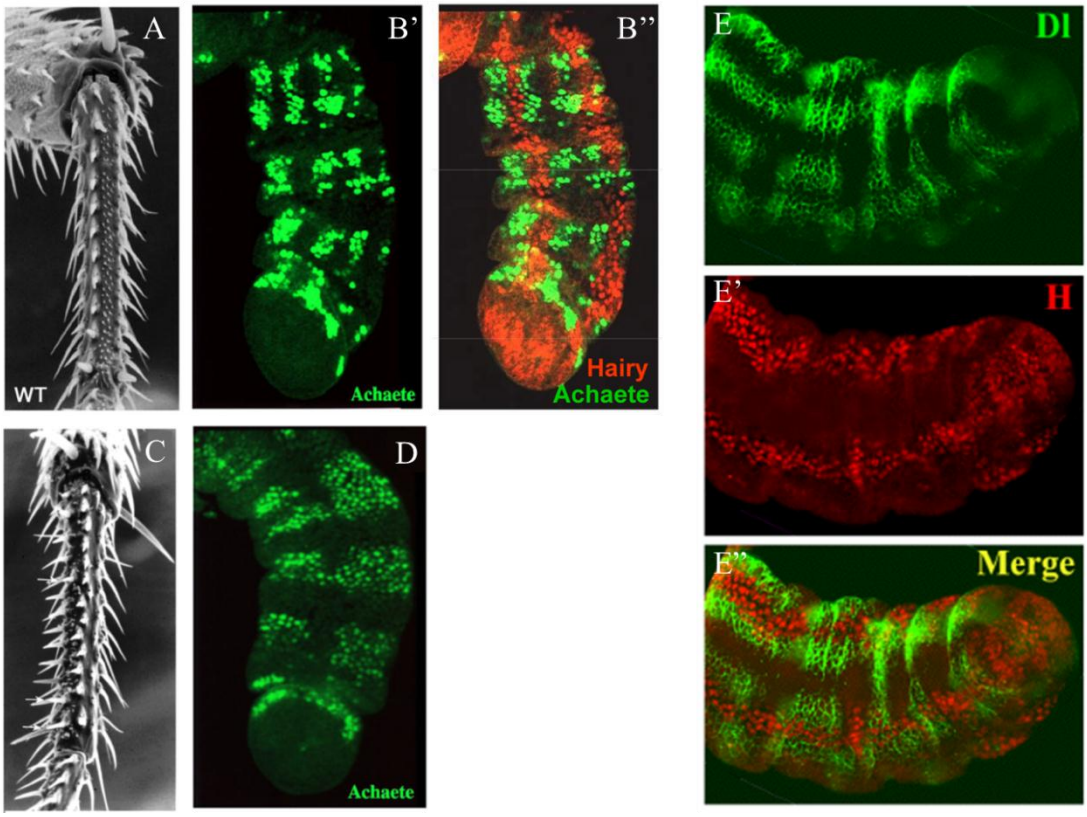
(A) Electron micrograph of the first tarsal segment, or basitarsus, of a wild type leg from the second thoracic segment (T2 leg). On the tarsal segments, the MS mCs are organized in eight longitudinal rows (L-rows; one is easily visible) along the leg circumference.

(B-B') Ac (visualized with anti-Ac, green) and Hairy expression (visualized with anti-Hairy, red) in a wild type mid-prepupal leg. Ac is expressed in eight narrow longitudinal stripes, which correspond to the mC proneural fields (four stripes are visible). The stripes of Ac expression are precisely positioned along the leg circumference. Hairy is expressed in four longitudinal stripes within four of eight Ac interstripe domains (two are visible), called the *hairy*-On interstripes.

(C) Electron micrograph of a T2 leg that lacks *hairy* function. Note the disorganization of the MS mC bristle rows.

(D) Ac expression in a *hairy* mutant mid-prepupal leg. In the absence of *hairy* function, Ac expression expands into the *hairy*-On interstripes, resulting in four broad stripes (two are visible) of Ac expression.

(E-E'') Dl (visualized with anti-Dl, green) and Hairy expression (visualized with anti-Hairy, red) in a wild type mid-prepupal leg. Dl is expressed in eight narrow longitudinal stripes, corresponding to the mC proneural fields (four stripes are visible) and precedes Ac expression. Hairy is expressed in four longitudinal stripes within four of eight Dl interstripe domains (two are visible).


A
WT
B'
Achaete
B''
Hairy
Achaete
C
D
Achaete
E
Dl
E'
H
E''
Merge

1.3.3.1. Role of *hairy* as a prepattern gene in regulating *ac* expression in the mC proneural fields

Figure 7A is a schematic representation of *ac* and *hairy* expression patterns drawn on the 3rd instar leg disc, for clear visualization. In the prepupal leg, *hairy* (*h*) is expressed in four longitudinal stripes (*hairy*-On interstripes, in red), each between two Ac stripes (proneural strips, in green), one pair that goes along the dorsoventral (D/V) axis (D/V-*h*) and another pair that runs along the anteroposterior (A/P) boundary (A/P-*h*). Each Hairy stripe is controlled by distinct enhancers that are responsive to Hh, Dpp and Wg signaling pathways (Hays et al., 1999; Kwon et al., 2004). D/V-*hairy* stripe is induced by Hh signaling near the A/P compartment boundary. Together with Hh, Dpp and Wg positively regulate the expression of *hairy* dorsally and ventrally, respectively, in its defined domain along the A/P boundary. The A/P-*hairy* stripe, however, appears to be activated broadly around the leg circumference and then repressed by Dpp dorsally and Wg ventrally to define the dorsal and ventral boundaries (Joshi et al., 2006).

hairy encodes a bHLH transcriptional repressor (Rushlow et al., 1989) and Hairy is known to function as a direct repressor of *achaete* in the wing by binding *ac* promoter (Van Doren et al., 1994; Ohsako et al., 1994). Hairy acts as a repressor of *ac* expression in these four *hairy*-ON interstripes in prepupal legs as well. In the absence of *hairy* function, *ac* expression expands to the regions where *hairy* is normally expressed and these broadened proneural fields results in disorganized bristle rows in the adult leg (Fig 6C and 6D) (Orenic et al., 1993). However, Hairy alone is not sufficient to regulate periodic *ac* expression in prepupal legs; only four of the eight interstripes are established by Hairy and the periodic *ac*

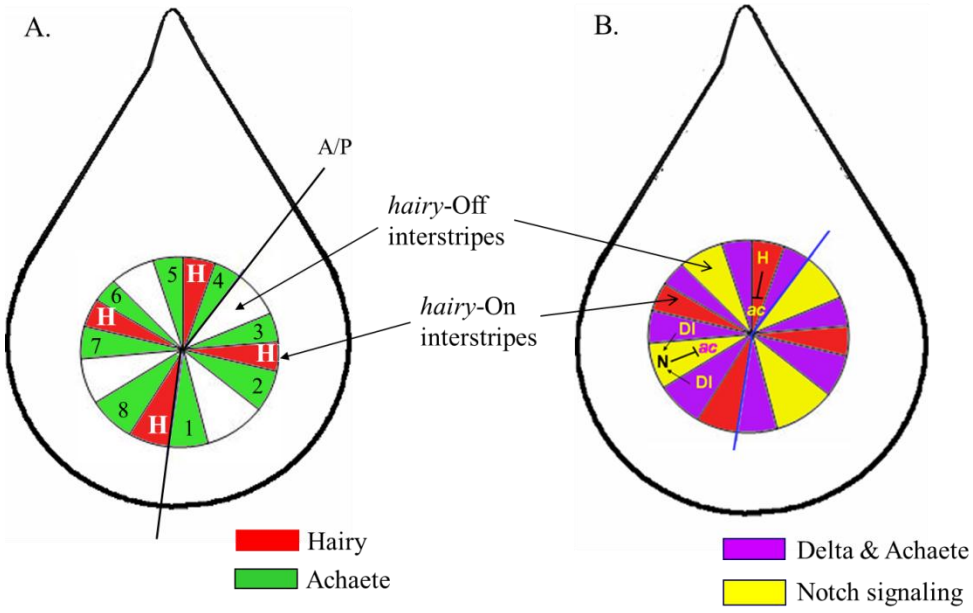


Figure 7. Schematic representation of Ac, Hairy and Dl expression in leg discs.

(A) Although Ac is not expressed before the mid-prepupal stage, Ac and Hairy expression are projected onto a 3rd instar disc to facilitate visualization. Ac is expressed in eight longitudinal stripes (proneural stripes, green), corresponding to the mC proneural fields. Hairy is expressed in four longitudinal stripes within four of eight *ac*-interstripe domains (*hairy*-On interstripes, red). The other four ac interstripes are referred to as the *hairy*-Off interstripes (white).

(B) Prior to the activation of *ac* expression, Dl is expressed in the mC proneural fields and eventually overlaps Ac (purple) expression. Dl activates Notch signaling in the *hairy*-Off interstripes (yellow).

expression is still established in the *hairy* mutant even though Ac stripes are broadened. Delta/Notch signaling has been identified as another prepattern regulator, in *hairy*-Off interstripes and this is discussed in the following section.

It has been shown that regulation of proneural gene expression by *hairy*-related bHLH prepattern regulators is conserved in the vertebrate central nervous system (Bae et al., 2005). In zebrafish, *hairy* homologue is *hairy- and enhancer of split-related (her)*. It has been reported that zebrafish *her3* and *her9* are expressed in the inter-proneural domains, which corresponds to *hairy*-On interstripes in fly, and repress the proneural gene expression in the inter-proneural domains, suggesting that *her3* and *her9* function as prepattern genes, which spatially defines the proneural gene expression.

1.3.3.2. Dual role of Delta/Notch signaling pathway in patterning of sensory organ and prepatterning of *ac* expression in the mC proneural fields

During the development of sensory bristles, cell-cell signaling mediated by the Notch (N) receptor is a crucial mechanism for cell fate decisions (reviewed by (Artavanis-Tsakonas et al., 1999). First, during lateral inhibition, N activity is required for restricting cell fates by singling out one or a few sensory organ precursors (SOP) from proneural clusters of *ac* and *sc* expressing cells. Later, N activity is also required for specifying the distinct cell fates for each of the four progeny cells through SOP cell divisions. The basic cell-cell signaling pathway mediated by N is as follows (reviewed by (Lai, 2004), Figure 8): N is activated by Delta (Dl), a transmembrane protein acting as a ligand for the N receptor.

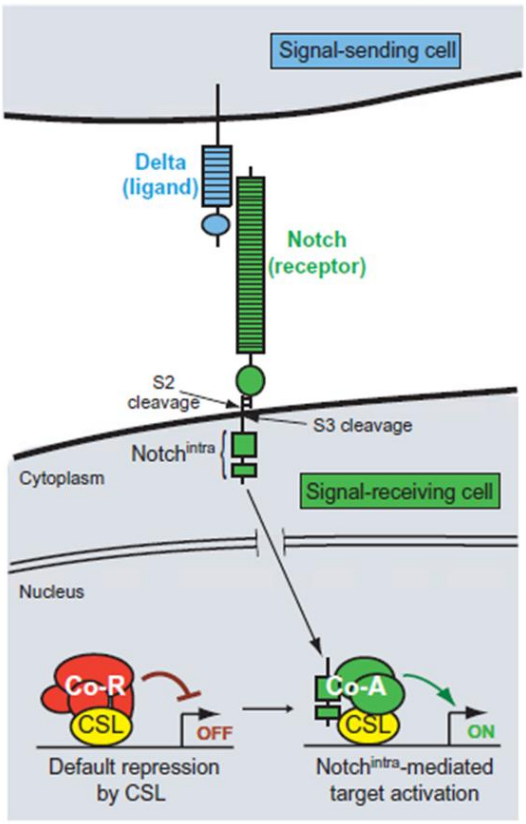


Figure 8. The Delta/Notch signaling pathway

This figure is taken from Lai et al., 2004. Upon binding the Delta ligand, the Notch receptor is cleaved through two proteolytic cleavages (S2 and S3). The Notch intracellular domain (NICD) then translocates to the nucleus and interacts with the transcriptional mediators CSL [CBF1/Su(H)/LAG1]. This binding displaces a CSL co-repressor complex (Co-R) and replaces it with a co-activator complex (Co-A), including NICD, and activates Notch target gene expression.

Upon activation, N is cleaved and N intracellular domain (NICD) translocates to the nucleus and collaborates with a sequence-specific DNA binding protein, Suppressor of Hairless [Su (H)] (Fortini and Artavanis-Tsakonas, 1994; Jarriault et al., 1995; Schweisguth and Posakony, 1992). Then, this Su (H)-NICD complex directly activates transcription of multiple N target genes including *Enhancer of split* Complex [E(Spl)-C] (Bailey and Posakony, 1995; Lecourtois and Schweisguth, 1995). Seven out of eleven *E(spl)*-C genes, *m3, m5, m7, m8, mδ, mγ, and mβ*, encode closely related bHLH repressor proteins (Delidakis and Artavanis-Tsakonas, 1992; Knust et al., 1992). The extensive genetic studies have found no lethal mutations in any of these genes, revealing the functional redundancy between *E(spl)*bHLH genes (Cooper and Bray, 1999; Delidakis et al., 1991). However, the individual *E(spl)*bHLH genes have distinct expression patterns (de Celis et al., 1996).

Together with *hairy*, Delta has been identified as another prepattern gene that defines periodic *ac* expression in the other four stripes (*hairy*-Off interstripes) (Joshi et al., 2006, Figure 7). Potential function of *Dl* as a prepattern regulator of proneural *ac* expression was suggested by studies in the notum (Parks et al., 1997). Within each notum, microchaete SOPs arise within proneural stripes between 10-12 hours APF and give rise to mechanosensory microchaetae which are organized in 10 longitudinal rows. When *Dl* function is reduced, Ac protein expression expands into interstripes in the notum, suggesting *Delta* function is required to define periodic *ac* expression in the microchaete proneural stripes. In the prepupal leg, *Dl* is expressed in eight longitudinal stripes overlapping Ac expressing stripes in the microchaete proneural fields (Joshi et al., 2006). In the *Dl* hypomorphic background (temperature sensitive alleles Dl^{RF}/Dl^{6B}), *ac* expression was expanded in the prepupal leg but *ac* expression does not expand into the *hairy*-On interstripes, suggesting that Dl is required

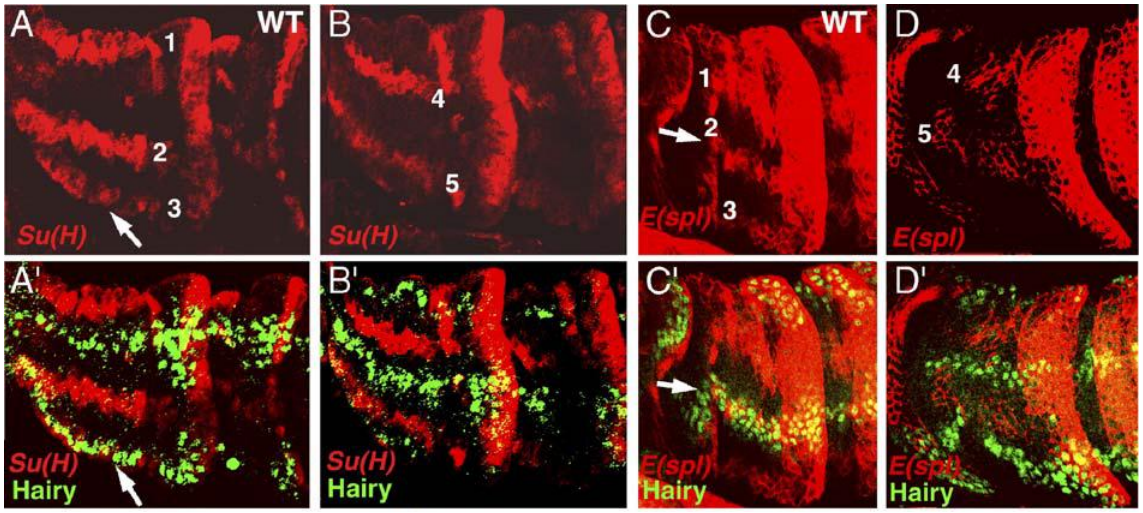


Figure 9. Delta/Notch signaling is activated in narrow domains complementary to the Hairy expression domains.

(A–B′) Comparison of Hairy (anti-Hairy, green in panels A′ and B′) and *Gbe+Su(H)m8-lacZ* expression (anti-β-Gal, red in panels A–B′) in a wild-type prepupal leg. Five *Gbe+Su(H)m8-lacZ* stripes are visible (numbered in panels A and B) in two focal planes. 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′ and D′) and *E(spl)mβ-CD2* expression (anti-CD2, red in panels C–D′) in a wild-type T2 prepupal leg. Five stripes of *E(spl)mβ-CD2* expression are visible (numbered in panels C and D) in two focal planes. 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).

for *ac* repression only in the *hairy*-Off interstripes. In loss of *N* function clones, ectopic *ac* expression was observed and when N signaling was widely induced, reduced *ac* expression was observed. These findings suggest that a role of Dl/N signaling in regulation of *ac* expression in the microchaete proneural fields (Joshi et al., 2006). It was determined that N signaling is activated in the *hairy*-Off interstripes by assaying two independent and widely expressed N-responsive reporters (Figure 9); The expression pattern of *Gbe+Su(H)m8-lacZ* (Fortini and Artavanis-Tsakonas, 1994) has been shown to accurately reflect N signaling in the wing. *E(spl)mβ,* one member of *E(spl)*-C genes, is the most widely expressed N-target gene (Bray and Furriols, 2001; Cooper et al., 2000; de Celis et al., 1996) and the expression pattern of *E(spl)mβ-CD2* reporter is similar to the pattern of *Gbe+Su(H)m8-lacZ* expression (Furriols and Bray, 2001). Both reporters are expressed in the region where N signaling is known to be activated in prepupal legs (Bishop et al., 1999; de Celis et al., 1998; Rauskolb and Irvine, 1999), suggesting that Dl/N signaling is required within the *hairy*-Off interstripes. From all these genetic data, it is suggested that high level of Dl expressed in the cells of the microchaete proneural fields (Ac stripes) signals to adjacent cells to activate Notch signaling in the *hairy*-Off interstripes, where *E(spl)*-C, Notch target genes, represses expression of proneural gene *ac* (Joshi et al., 2006)

1.3.4. Model for patterning of leg mechanosensory microchaete bristles

The genetic studies of our laboratory allow us to outline a general genetic pathway for the regulation of *ac* expression in the leg mC proneural fields (Figure 10). Periodic *ac* expression for microchaete primordia is established progressively. During the early 3rd larva

instar leg disc development, *hairy* expression begins to appear in the longitudinal stripes; first, D/V-*hairy* stripes, which are regulated by global patterning molecules such as Hh, Dpp and Wg, are established and then between 3 and 4 hours APF the A/P-*hairy* stripes are expressed. Between 6 and 7 hours APF, *Dl* expression is established within the mechanosensory microchaete primordia. Then, *ac* expression can be broadly activated, however, it is spatially defined into eight narrow stripes by four Hairy stripes and Dl/N signaling which are established earlier (Joshi et al., 2006).

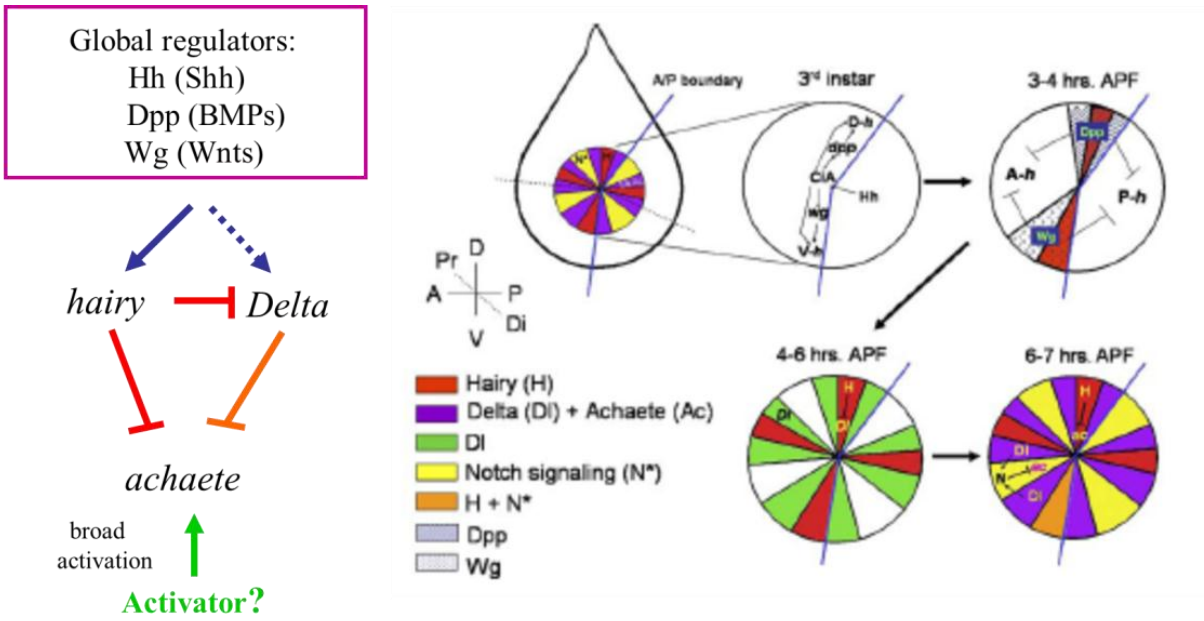


Figure 10. Model for *Drosophila* leg mC patterning.

Expression of proneural gene *ac* is broadly activated but is spatially defined by two prepattern regulators, Hairy and Dl. *hairy*, and perhaps *Dl*, expression, is regulated by global regulators of leg development, Hh, Dpp, and Wg. The *ac* proneural pattern is established progressively. Expression of the prepattern regulators that control *ac* expression, Hairy and Dl, is established prior to *ac* expression. First, two Hairy stripes are expressed along the D/V axis (during 3rd instar), followed by another two stripes along the A/P axis (at 3-4 h APF). Then, Dl expression is established between 4-6 h APF. After the repressive prepattern is established, *ac* expression is activated (6 h APF).

II. MATERIALS AND METHODS

2.1. *Drosophila* strains

The following *Drosophila melanogaster* strains were used in this research: OregonR, UAS-*NICD*; *Cyo* (Kidd et al., 1998), *w;* Df/*Cyo*$^{vg\text{-}lacZ}$; UAS-*NICD*/ Tm6 (Hu), gift from Katzen Lab, UAS-*hairy*, *rn*-Gal4/Tb, *In(1)sc*^{8}, *In(1)sc*^{v2}, Df(1)*sc*$^{10\text{-}1}$, Df(1)*y*^{3PL}*sc*^{8R}.

Transgenic fly lines were generated for this research and their genotypes are listed in Table 1.

Table 1. Transgenic fly lines generated for this research

Transgenic fly stock	Genotype of stock
ac-Res1 (12kb- *ac* rescue)	*w; +/+;* P1-*ac-Tm3/rydp* Df(1) *y3PL* sc*8*; P1/+; P1/+
ac-Res3 (P4- *ac* rescue)	Df(1) *y3PL* sc*8*; PG4/cyo; +/+
ac-Res2 (P3-*ac* rescue)	Df(1) *y3PL sc8*, +/+, P3-*ac* / *Tb*
ac-GFP-FL (*ac* mC-CRE-GFP reporter)	*w*; +/+, *ac* mC-CRE-*GFP* / *Tb*
ac-Res-FL (*ac* mC-CRE rescue)	Df(1) *y3PL* sc*8*; *ac* mC-CRE-*ac /Cyo* Df(1) *y3PL* sc*8*; +/+/, *ac* mC-CRE*/Tb*
ac-GFP-Act (*ac* mC-CRE-Act-GFP reporter)	*w;ac* mC-CRE-Act GFP*; Cyo/+;+/+*
ac-Res-Act (*ac* mC-CRE-Act rescue)	*w;ac* mC-CRE-Act-*ac/ Cyo* *w;ac* mC-CRE-Act-*ac/Tb*
ac promoter -*GFP*	*w; ac* promoter-*GFP/Sb*
ac promoter - *ac*	*w*; *ac* promoter - *ac /Cyo; +/+* *w; +/+; ac* promoter-*ac/Sb*
ac-GFP-Act-H1* (*ac* mC-CRE-Act-GFP with mutated H site in *ac* promoter	*w*; *ac*-GFP-Act-H1* */Cyo; +/+*
ac-Res-Act-H1* (*ac* mC-CRE-Act-*ac* with mutated H site in *ac* promoter)	*w; ac*-Res-Act-H1* */Cyo;+/+* *w; ac*-Res-Act-H1* */Cyo; ac*-Res-Act-H1 */Tb*
ac-GFP-FL-H1* (*ac* mC-CRE-GFP with mutated H site in *ac* promoter)	*w; ac*-GFP-FL-H1* */Cyo; +/+* *w;+/Cyo; ac*-GFP-FL-H1* */Tb* *w/ ac*-GFP-FL-H1**;/+/+;+/+*
ac-Res-FL-H1* (*ac* mC-CRE-ac with mutated H site in *ac* promoter)	*w; ac*-Res-FL-H1* */Cyo; ac*-Res-FL-H1* */Tb*
ac-GFP-FL-H1*H2* (*ac* mC-CRE-GFP with double mutation on H sites)	*w; ac*-GFP-FL-H1*H2* */Cyo; +/+*

Table 1. Transgenic fly lines generated for this research (continued)

ac-Res-FL-H1*H2* (*ac* mC-CRE-*ac* with double mutation on H sites)	*w; ac*-Res-FL-H1*H2* */Cyo; +/+* *w;+/+; ac*-Res-FL-H1*H2* */Tb*
ac-GFP-FL-E* (*ac* mC-CRE-GFP with mutation of E(spl) site in the mC-CRE)	*w; ac*-GFP-FL-E* */Cyo; +/+*
ac-Res-FL-E* (*ac* mC-CRE-ac with mutation of E(spl) site in the mC-CRE)	*w; ac*-Res-FL-E* */Cyo; +/+* *w;+/+; ac*-Res-FL-E* */Tb*
ac-GFP-FL-H1*H2*E* (*ac* mC-CRE-GFP with triple mutation on Hairy and E(spl) sites)	*w; ac*-GFP-FL-H1*H2*E* */Cyo; +/+*
ac-Res-FL-H1*H2*E* (*ac* mC-CRE-ac with triple mutation on Hairy and E(spl) sites)	*w; ac*-Res-FL-H1*H2*E* */Cyo; +/+* *w;+/+; ac*-Res-FL-H1*H2*E* */Tb*
ac-GFP-Act-H*+H (*ac* mC-CRE-Act-GFP with mutated H site in the *ac* promoter + *ac* mC-CRE H binding site)	*w; ac*-GFP-Act-H*+H */Cyo; +/+*
ac-GFP-Act-H*+E (*ac* mC-CRE-Act-GFP with mutated H site in the *ac* promoter + E(spl) binding site)	*w; ac*-GFP-Act-H*+E */Cyo; +/+* *w;+/+; ac*-GFP-Act-H*+E */Tb*

2.2. Genetics

For mC bristle rescue analysis, adult legs of all transgenic flies carrying the rescue construct were examined in *Df*(1)*sc*$^{10\text{-}1}$ background and each genotype can be represented as *Df*(1)*sc*$^{10\text{-}1}$; transgene. For the mounting of adult leg, notum and wing, adult flies were dehydrated by serially incubating in ethanol concentrations of 70%, 80%, 90%, 95%, and 100% for 5 minutes each at room temperature. Legs were dissected, mounted in Gary's magic mountant, and dried at 65 °C overnight.

For analysis of *ac* expression pattern in mC proneural fields, prepupal legs carrying transgene in Df(1) *y3PLsc8* background were dissected at 6 h APF and stained with antibodies, anti-Ac and anti-Hairy. The genotypes of flies used can be represented as *Df(1) y3PL sc8*; rescue transgene, e.g. Df(1) *y3PL sc8; ac*-Res-FL.

For ectopic expression studies, prepupal legs carrying GFP reporter constructs were dissected from the genotypes which can be represented as the followings; UAS-*hairy*/ transgene; *rn*-Gal4/+, UAS-*hairy*/+; *rn*-Gal4/ transgene, UAS-*NICD*; transgene/+; +/+, transgene/+; UAS-*NICD*/ *rn*-Gal4, and transgene; UAS-*mCherry*/+; *rn*-Gal4/+.

2.3. Generation of transgenic flies

2.3.1. Preparation of DNA rescue constructs

In order to generate the rescue constructs, the wild type genomic DNA fragments were cloned into the pCasper4 vector, which is commonly used for *Drosophila* transformation (Thummel and Pirrotta, 1992). For the *ac* mC-CRE rescue construct**,** a 2.2kb *Eco*RI fragment, which includes *ac* promoter and *ac* coding region with a 700bp sequence 3' of the *ac* coding region were cloned into the pCasper4 vector. The 4kb *Xba*I/*Kpn*I fragment was cloned 5′ to the 2.2kb *Eco*RI fragment as a *Not*I/*Kpn*I. For *ac* mC-CRE-Act rescue construct, the 600bp *Sal*I/*Bam*HI fragment was cloned 5′ to the 2.2kb *Eco*RI fragment as a *Xba*I/*Kpn*I fragment.

The QuikChange Site-Directed Mutagenesis Kit (Stratagene) was used for point mutations on Hairy and E(spl) sites as described below. The mutagenized sequences are listed in Table 2. The 2.2kb *Eco*RI fragment was cloned into pBS and the Hairy site in *ac* promoter was mutated (CACGCG to CTTTCG), and then cloned back into the *ac* mC-CRE rescue construct. For mutagenesis of E(spl) and Hairy site in the *ac* mC-CRE, 4kb *Sal*I fragment was cloned into pBS and mutated. For the Hairy site, the same mutation was made as in *ac* promoter and for the E(spl) site, the core consensus sequence CACGTG was mutated to CATTTG and GGTTAA. The 4kb fragment with mutations, individually and together, was cloned back into the rescue construct as *Xho*I/*Not*I fragment with and without mutated Hairy site in *ac* promoter. For the *ac* mC-CRE-Act construct, the 600bp *Sal*I/*Bam*HI fragment taken from the *ac* mC-CRE rescue construct was cloned into pBS and then cloned back 5' of 2.2kb *Eco*RI fragment with and without mutated Hairy site as *Xho*I/*Not*I fragment.

Table 2. Mutagenized sequences (mutated bases are shown in lower case in red)

Site mutated	Wild type sequence	Sequence after mutagenesis
Hairy binding site in *ac* promoter	GGCCACGCGAC	GGCCtttCGAC
Hairy binding site in *ac* mC-CRE	GGCCACGCGAC	GGCCtttCGAC
E(spl) binding site in *ac* mC-CRE	TGGCACGTGGCC	TGGggttaaGCC

2.3.2. Preparation of DNA GFP reporter constructs

In order to generate the GFP reporter constructs, the DNA fragments were cloned 5′ to the *ac* promoter in the pStinger GFP vector (Barolo et al., 2000). For the *ac* mC-CRE GFP construct, the *ac* promoter was cloned into the pStinger vector as a *Kpn*I/*Nhe*I fragment and the 4kb *Sal*I genomic fragment was cloned 5′ to the *ac* promoter as a *Xba*I/*Kpn*I fragment. For *ac* mC-CRE-Act GFP construct**,** the 600bp *Sal*I/*Bam*HI fragment was cloned 5′ to the *ac* promoter as a *Pst*I/*Kpn*I fragment.

The GFP constructs with mutations were generated as follows: For mutagenesis of Hairy site in the *ac* mC-CRE**,** the 2.2kb *Eco*RI fragment with a mutated Hairy site, which is cloned in pBS, and the *ac* mC-CRE GFP construct was digested with *Kpn*I and *Not*I. The 2.2kb fragment with a mutated Hairy site was ligased with the *ac* mC-CRE-GFP fragment which is missing 2.2kb, with and without mutated Hairy site. For mutagenesis of the E(spl) site, the 4kb *Sal*I with a mutated E(spl) site was taken from the *ac* mC-CRE rescue

construct as a *Pst*I/*Xho*I fragment and cloned to 3' of *ac* promoter in pStinger vector. For the *ac* mC-CRE-Act GFP construct with a mutated Hairy site, the 600 *Sal*I /*Bam*HI in pBS was cloned into pCasper vector as a *Xho*I/*Kpn*I fragment. This piece was cloned into 3' of *ac* promoter with a mutated Hairy site in pStinger vector as *Pst*I/*Kpn*I fragment.

For the functional analysis of Hairy and E(spl) sites in the *ac* mC-CRE, a synthetic oligonucleotides was designed for each site and inserted as a KpnI/EcoRI fragment, 3' of *ac* mC-CRE-Act fragment in the GFP construct of *ac* mC-CRE-Act with a mutated Hairy site.

The sequences used for a synthetic Hairy binding site is as follows: (core binding sequence is shown in red)

5'- CAATTGCCACTGCGCACGCGCACGCCCAC-3' and

5'-AATTCTGGGCGTGCGCGTGCGCAGTGGCAATTGGTAC-3'

The sequences used for a synthetic E(spl) binding site is as follows: (core binding sequence is shown in red)

5'-CTCCTTTTTTGCACGTGGCCCAAGAATAAG-3'

5'-AATTCTTATTCTTGGGCCACGTGCAAAAAAGGAGGTAC-3'

2.3.3. Site-specific mutagenesis

For mutagenesis of all the rescue and GFP reporter constructs, the QuikChange Site-Directed Mutagenesis Kit (Stratagene) was used. The primers containing the point mutations of Hairy and E(spl) site were designed using Vector NTI (Invitrogen) for PCR reactions. Following PCR reactions using *PfuTurbo*® DNA polymerase, PCR product was treated with

*Dpn*I to remove original plasmid templates and was transferred to the *E.coli* DH5α competent cells for transformation. The mutated sites were confirmed by automatic sequencing (UIC Research Resource Center). The DNA constructs for microinjection were purified using HiSpeed Plasmid Midi Kit (Qiagen).

2.3.4. Germline transformation

The prepared DNA constructs were injected into the *w*; Δ2-3 flies and transgenic flies were generated by P-element mediated germline transformation (Rubin and Spradling, 1982).

2.4. Immunohistochemistry and microscopy

For all antibody stainings, prepupal legs were dissected between 4-6 h APF and treated as described (Carroll and whyte, 1989). Primary antibodies used includes: mouse-anti-Achaete, 1:10 (Skeath and Carroll, 1991), mouse-anti-Hairy, 1:4 or 1:5 and rabbit anti-Hairy, 1:200 (Carroll et al., 1988), and mouse anti-N intracellular domain (NICD), 1:250, from the Developmental Studies Hybridoma Bank.

All fluorescent images were collected on Zeiss Axiovert 200M Microscope equipped with ApoTome and a digital camera. Images were collected as apotomized Z-stacks and were subjected to 3D-deconvolution.

III. ANALYSIS OF *cis*-REGULATORY ELEMENTS THAT REGULATE *achaete* EXPRESSION IN THE MECHANOSEONSORY MICROCHAETE PRONEURAL FIELDS OF THE *DROSOPHILA* LEG

3.1. Introduction

During vertebrate and invertebrate development, components of nervous system must be precisely patterned. How this precise pattern is established during development has been one of the most fundamental and interesting biological questions. The purpose of this research is to understand the genetic and molecular mechanisms underlying the pattern formation of sensory organs, which comprise the peripheral nervous system (PNS). The *Drosophila melanogaster* leg is a good model system to study pattern formation of the PNS because they bear many innervated sensory bristles that are precisely organized in a stereotyped pattern. Patterning of sensory bristles requires spatially defined expression of the redundant proneural genes, *achaete* (*ac) and scute* (*sc*), which encode basic-helix-loop-helix (bHLH) transcriptional activators. The *ac* and *sc* genes are members of the *ac/sc* complex (ASC), shown in Fig. 11. *ac* and *sc* are identically expressed and function redundantly to specify a sensory organ fate in the adult PNS. Ac and sc confer on the cells the potential to become sensory organ precursors (SOPs). SOP development depends on expression of *ac* or *sc* in groups of cells, called proneural clusters, located at precisely determined positions. From these proneural clusters, one or a few SOPs are selected (Romani et al., 1989; Cubas et

al., 1991; Skeath and Carroll, 1991). Expression of *ac* and *sc* in proneural clusters in the adult primordia is established by a group of prepattern regulators that control *ac/sc* expression.

Previous extensive studies on pattering of the *Drosophila* macrochaete (MCs) in the dorsal thorax, called the notum, have shown that *ac* and *sc* expression in primordia of early specified bristles is induced at specific positions within the notal region of the 3rd instar wing imaginal disc and is controlled by several discrete shared *cis*-regulatory elements (MC-CREs) (Campuzano and Modolell, 1992; reviewed in Gomez-Skarmeta et al., 2003). Compromised function of a specific CRE results in loss of both *ac* and *sc* expression in the primordium of the corresponding MC bristle and loss of the bristle in the adult. The MC-CREs are spread over 100 kb throughout the *ac-sc* gene complex. Each MC-CRE responds to local positional regulators to establish proneural gene expression in corresponding proneural cluster.

Two types of sensory bristles, based on function, found on the leg include: mechanosensory (MS) bristles, macrochaetae (large) and microchaetae (mC, small), and chemosensory microchaetae (CS mC). These sensory bristles can also be classified according to their specification time: early specified and late specified bristles. The MS MCs and CS mCs are specified during the 3rd larval instar stage (early), while the MS mCs are specified during the mid-prepupal stage (late). Prepupal development takes place between 0 to 12 hours (h) after puparium formation (APF). Drosophila legs are segmented along the proximal/distal (P/D) axis and within each leg segment, the MS mCs are organized in a series of precisely positioned longitudinal rows along the leg circumference. From this point, mCs will refer to the MS mCs, unless otherwise indicated.

Genetic studies in our laboratory have allowed us to uncover a general genetic pathway for the regulation of *ac* expression in the leg mC proneural fields (Joshi et al., 2006).

In mid-prepupal legs, *a*c is expressed in eight longitudinal stripes, corresponding to the the mC proneural fields, that alternate with eight interstripe domains. *ac* expression in the mC proneural field is controlled by two prepattern regulators, Hairy and the Notch (N) ligand, Delta (Dl), both of which are periodically expressed prior to activation of *ac*. *ac* expression in the mC proneural fields is established progressively. First, the bHLH repressor, Hairy, is expressed in four of the eight *ac* interstripe domains, referred to as the *hairy*-ON interstripes. Two of the *hairy* stripes are expressed along the dorsal/ventral axis (D/V-*hairy* stripes), and a second pair of *hairy* stripes is expressed along the anterior/posterior axis (A/P-*hairy* stripes). The D/V-*hairy* stripes are expressed in early 3rd instar legs, while A/P-*hairy* expression begins at 3 hrs. APF. *Dl* expression is activated between 4-5 hrs. APF in eight stripes corresponding to the mC proneural fields, which begin expressing *ac* by 6 hrs. APF. Dl signals specifically to cells in the *hairy*-OFF interstripes, activating N signaling, which we hypothesize represses *ac* expression via one or more of the bHLH repressors of the Enhancer of Split complex (Orenic et al., 1993; Joshi et al., 2006).

Our observations indicate that patterning of early-specified MCs and the late specified mCs differ in key ways. Proneural gene expression in the MC primordia is activated locally at specific positions in the notal disc and is controlled by several modular enhancers that respond to locally expressed activators and repressors. On the other hand, our studies suggest that expression of *ac* in the leg mC proneural fields involves global activation of *ac* expression along the leg circumference combined with Hairy and Dl/N-mediated repression. The goal of my research has been to test this model. In this chapter, we report that, unlike the notal MC primordia, *ac* expression in the leg mC primordia can be established by

the activity of a single CRE that can direct *ac* expression in all the mC primordia and that is responsive to Hairy and Dl/N mediated repression.

3.2. Localization of *cis*-regulatory elements responsible for periodic expression of *achaete* in the mechenosensory microchaete proneural fields in the *Drosophila* prepupal leg

Our previous investigations suggest a model for specification of mC bristles that involves broad activation of *ac* expression combined with refinement via the activity of two repressors, Hairy and Delta (Orenic et al., 1993; Joshi et al., 2006). This mechanism results in establishment of eight narrow proneural fields along the leg circumference in prepupal legs by 6 h APF from which the mC sensory organ precursors (SOPs) will be selected.

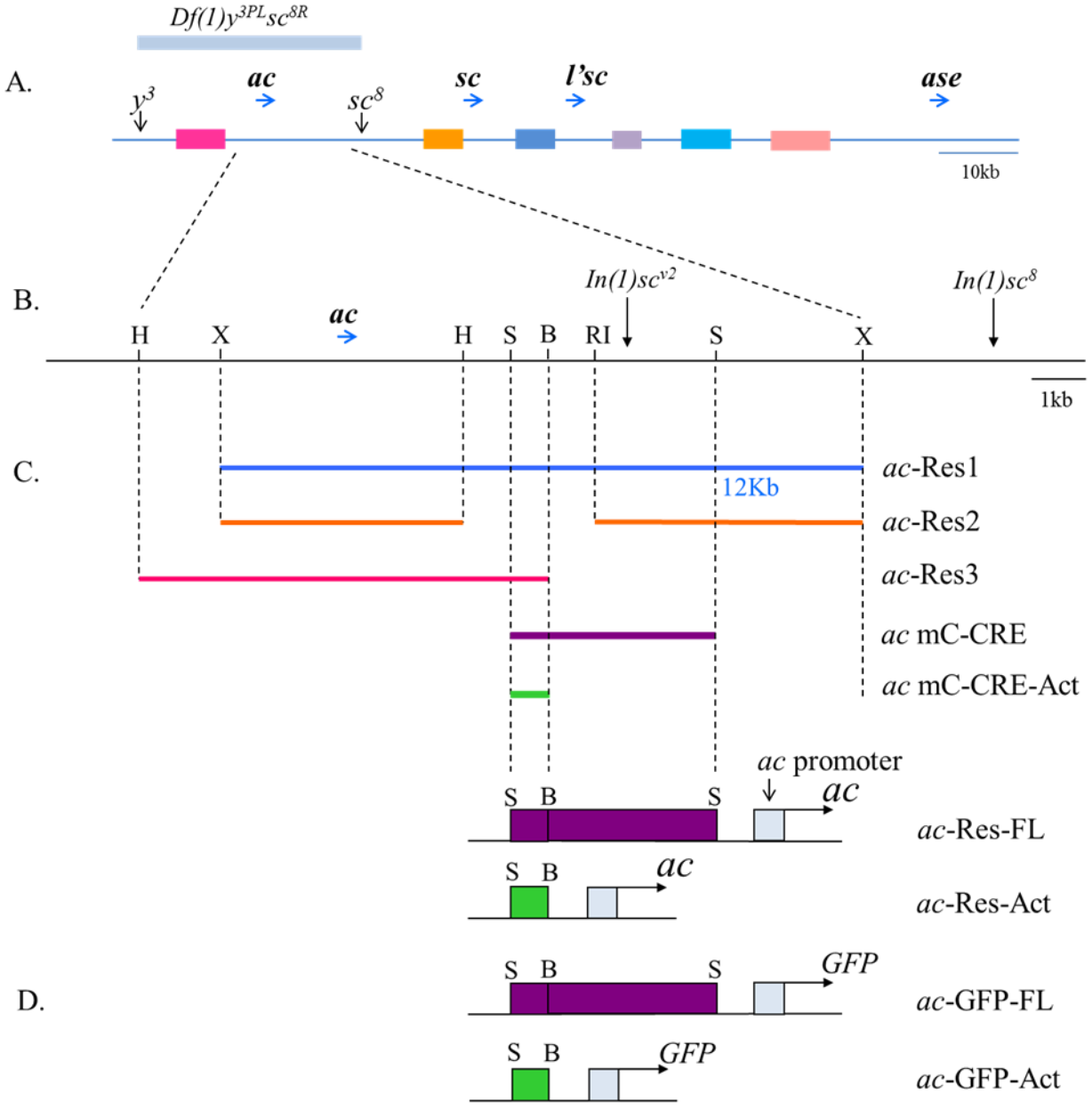
As a test for this model, we sought to identify the CREs that control *ac* expression in the mC proneural fields. To narrow down their location, we examined *ac* expression pattern in legs carrying breakpoint mutations in the ASC. The ASC is on the X chromosome, and many mutations, including inversions and deletions, in the complex are viable in hemizygous males. For this study, we examined expression in prepupal legs from *In(1)sc*^{v2}/Y and *In(1)sc*^{8}/Y males (Figure 11B).

In(1)sc^{v2} (Campuzano et al., 1985), is an inversion with a breakpoint between *Bam*HI and *Eco*RI restriction sites downstream of the *ac* transcription unit (Fig. 11B). Prepupal legs from *In(1)sc*^{v2}*/Y* animals exhibit loss of Ac expression in the distal half of each tarsal segment and expansion of expression along the leg circumference into *hairy*-Off interstripes

Figure 11. Schematic maps of *ac/sc* complex and genomic fragments tested in rescue and reporter constructs.

(A) The *ac/sc* complex of *Drosophila* consists of four genes, *achaete (ac), scute (sc), lethal of scute (l'sc),* and *asense (ase)*. The rectangular boxes, distributed throughout the *ac-sc* complex, represent previously identified *cis*-regulatory elements, which direct proneural gene expression in the primordia of large bristles, macrochaete, and are shared by *ac* and *sc* (Gomez-Skarmeta et al., 1995; reviewed in Calleja et al., 2002).

(B-D) Map of the *ac* region, enlarged in (B), showing the genomic fragments tested in transgenic rescue (potential CREs, *ac* promoter, and *ac* coding region), shown in (C), and reporter (potential CREs, *ac* promoter and GFP) constructs, shown in (D). *ac*-Res1-3 are rescue constructs consisting of the *ac* gene and varying lengths of adjacent sequence. In *ac*-Res-FL and *ac*-Res-Act, putative CREs were cloned 5' to a minimal *ac* gene consisting of the *ac* promoter and transcription unit (C). These putative CREs were also cloned 5' to a reporter gene consisting of the *ac* promoter and GFP gene (D). The 4kb *Sal*I fragment, corresponding to the putative microchaete enhancer (*ac* mC-CRE), was cloned into a rescue construct consisting of the *ac* promoter and *ac* coding region or a reporter construct with the *ac* promoter and *GFP* gene. Arrows in panels A and B indicate breakpoints. B: *Bam*HI, H: *Hind*III, RI: *Eco*RI, S: *Sal*I, X: *Xba*I.


A.
Df(1)y3PLsc8R
y3
ac
sc8
sc
l'sc
ase
10kb
B.
In(1)scv2
In(1)sc8
H
X
ac
H
S
B
RI
S
X
1kb
C.
ac-Res1
12Kb
ac-Res2
ac-Res3
ac mC-CRE
ac mC-CRE-Act
ac promoter
ac
S
B
S
ac-Res-FL
ac-Res-Act
GFP
ac-GFP-FL
ac-GFP-Act
D.

(Figure 12A-A'). This observation suggests that *ac* expression in the leg mC primordia is disrupted, although not completely eliminated by this mutation. Furthermore, it appears that this mutation disrupts response to Dl/N-mediated repression of Ac. These findings imply that *ac* mC CREs is disrupted and may map close to the $In(1)sc^{v2}$ breakpoint. However, $In(1)sc^{8}$ (Campuzano et., 1985), an inversion breakpoint further downstream of $In(1)sc^{v2}$, does not disrupt *ac* expression in prepupal legs (Figure 12B-B'), suggesting that *ac* mC CREs are located 5' to the $In(1)sc^{8}$ breakpoint.

In order to narrow down the location of a putative *ac* mC CRE required for *ac* expression in the mC proneural fields, we generated transgenic flies carrying an *ac*-minigene construct, a 12 kb *Xba*I fragment (*ac*-Res1), that spans the region from 3 kb upstream to 7 kb downstream of the *ac* gene (Figure 11C). These transgenic flies were crossed into a $Df(1)sc^{10\text{-}1}$ background and we examined the microchaete rescue phenotype. Two molecular lesions are associated with $Df(1)sc^{10\text{-}1}$, a deletion of the *ac* gene and a nonsense mutation in *sc*, and therefore, $Df(1)sc^{10\text{-}1}$ lacks both *ac* and *sc* function. $Df(1)sc^{10\text{-}1}$/Y males lack most sensory bristles on the notum and legs (Figure 13B and 14B) (Campuzano et al., 1985; Villares and Cabrera, 1987). The *ac*-Res1 transgene rescues almost all the mechanosensory microchaete (MS mC) on the legs (Figure 13D) and notum (Figure 14C), but none of mechanosensory macrochaete (MS MC) or chemosensory bristles on either the leg or the notum were rescued. This observation suggests that the *ac*-Res1 transgene functions specifically to rescue late-specified sensory bristles but not early specified bristles. One interpretation of this observation is that the *ac*-Res1 transgene lacks sequences necessary to express *ac* at high enough levels to confer rescue of early-specified sense organs. Alternatively, it is plausible that sequences contained within the *ac*-Res1 transgene confer

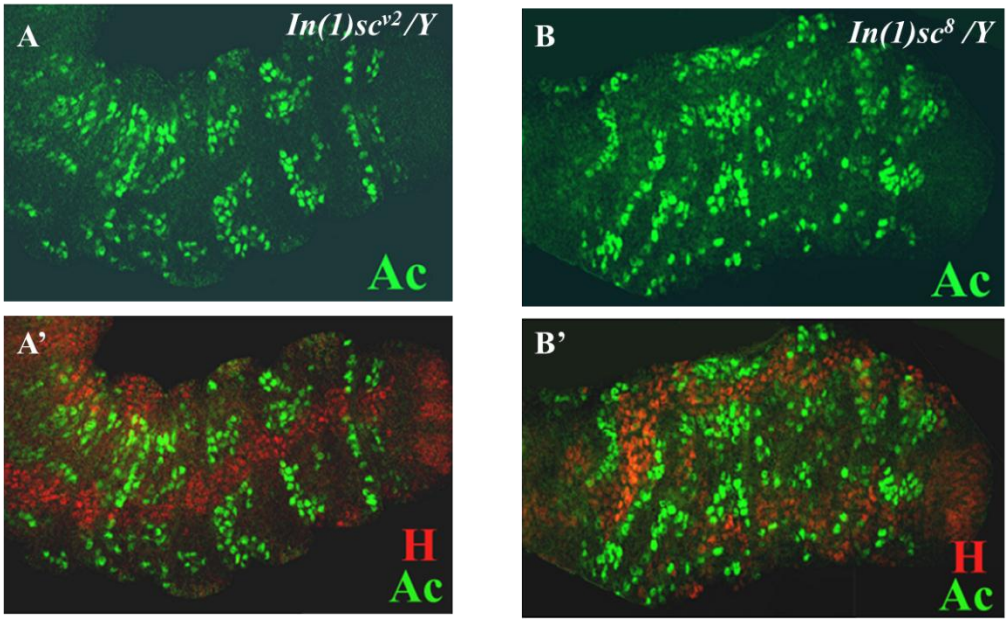


Figure 12. Ac expression in prepupal legs carrying breakpoint mutations in the *ac/sc* region.

(A-A) Expression of *ac* (anti-Ac, green) and *hairy* (anti-Hairy, red) in a *In(1)sc*^{v2}/Y leg. Ac expression looks expanded and disrupted.

(B-B") Expression of *ac* (anti-Ac, green) and *hairy* (anti-Hairy, red) in an *In(1)sc*^{8}/Y leg. Ac is expressed in narrow stripes similar to wild type expression.

temporal control of *ac* expression, such that *ac* is only expressed during the late stages when the MS mC are specified. To distinguish between these possibilities, we crossed in 3 copies of the *ac*-Res1 transgene into a $Dfsc^{10\text{-}1}$. We find that, even 3 copies of the transgene fails to confer rescue of early specified sense organs in the legs, although we occasionally observe rescue of one MC in the notum. This observation combined with expression analysis (see below) is consistent with the hypothesis that the *ac*-Res1 transgene directs late expression of *ac*. It is notable that even though the sequence contained within the *ac*-Res1 rescue construct are lacking in the $Df(1)y^{3PL}sc^{8R}$ deficiency, legs from $Df(1)y^{3PL}sc^{8R}$ /Y flies form the sensory bristles in the notum and the leg. Moreover, they exhibit the wild type mC pattern in the legs, implying that there is at least one additional CRE, which can drive *sc* expression in the leg mC primordia.

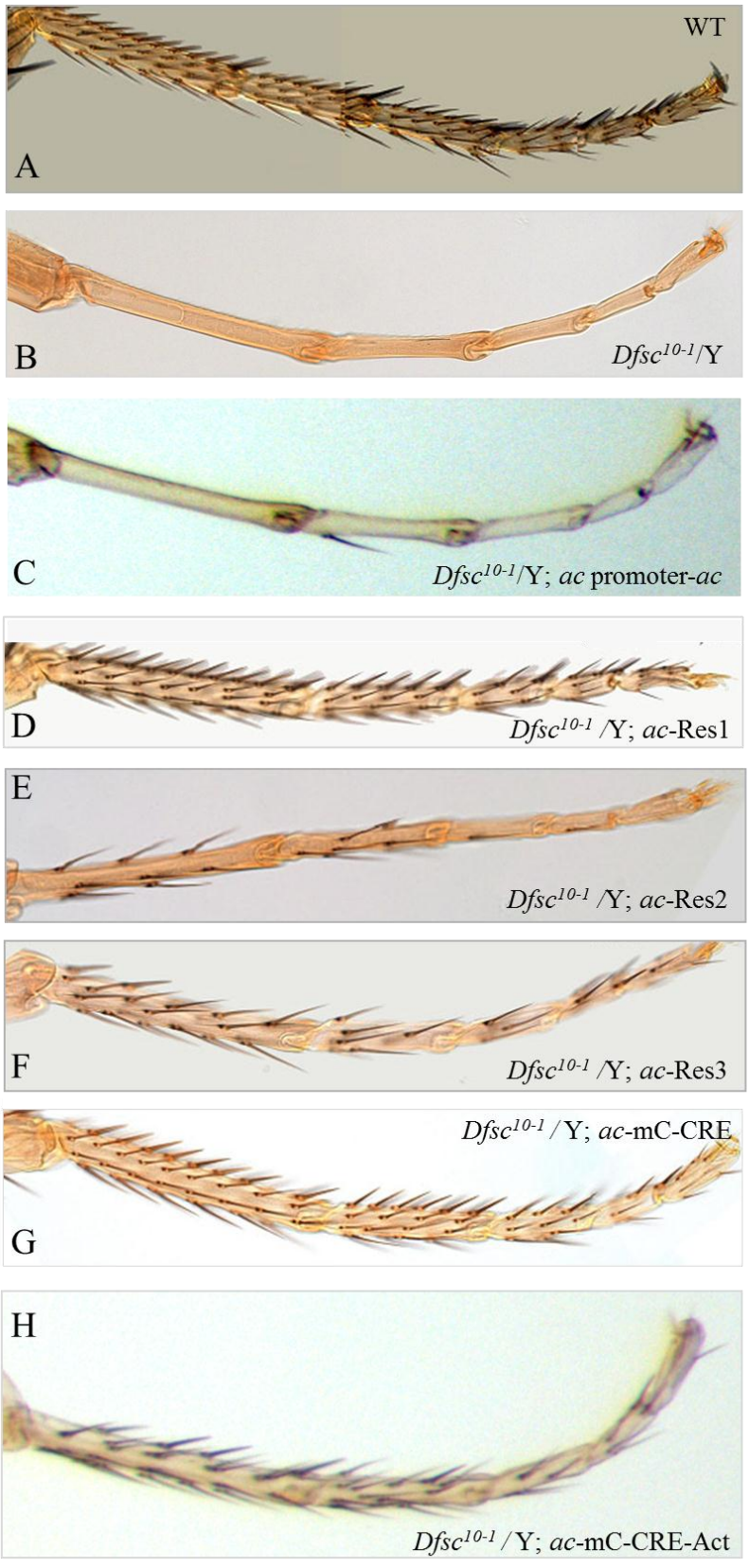
We also examined the expression of *ac* driven by *ac*-Res1 transgene in a $Df(1)y^{3PL}sc^{8R}$ background using an anti-Ac antibody. $Df(1)y^{3PL}sc^{8R}$ flies lack the *ac* coding region and all other sequences contained within the P1 transgene, while the *sc* coding sequences are intact (Skeath et al., 1992). The 12kb *ac*-Res1 fragment in $Df(1)y^{3PL}sc^{8R}$ background directs *ac* expression in the eight longitudinal mC proneural stripes in the prepupal leg at 6 h APF (Figure 15D), but no *ac* expression is observed in 3rd instar leg discs. This suggests that the *ac*-Res1 transgene contains all the necessary elements for the generation of the MS mC in a periodic pattern and is specific for the MS mC formation. Together, these results suggest that the sequences contained within the *ac*-Res1 transgene are sufficient to direct the correct spatial and temporal pattern of *ac* expression in the leg mC proneural fields and for formation of mCs in adult legs. Furthermore, our observations imply

Figure 13. Phenotypes of adult legs from Df*sc*$^{10\text{-}1}$/Y males carrying different rescue transgenes.

(A) Wild type *Drosophila* T2 leg exhibiting all three types of sensory bristles, the mechanosensory macrochaete (MS MC), the chemosensory microchaete (CS mC), and the mechanosensory microchaete (MS mC).

(B) An adult *Df(1)sc*$^{10\text{-}1}$ /Y leg, which does not make functional Ac and Sc, exhibits no sensory bristles.

(C-H) Adult *Df(1)sc*$^{10\text{-}1}$*/Y* legs carrying different transgenic rescue constructs: (C) a transgene consisting of the *ac* promoter + the *ac* gene does not rescue MS mCs in *Df(1)sc*$^{10\text{-}1}$*/Y* legs. (D) The *ac*-Res1 transgene rescues almost all the MS mC, but not the CS mC or the MCs. (E-F) The *ac*-Res2 transgene rescues a few MS mC in the proximal region of each tarsal segment (E) The *ac*-Res3 transgene preferentially rescues MS mCs in proximal region tarsal segments (F). (G) The *a*c-Res-FL transgene, containing the 4kb *Sal*I fragment, which corresponds to the *ac* mC-CRE, cloned 5' to the *ac*-promoter + *ac* gene, rescues almost all the MS mC, but not CS mC or MS MC. (H) The *ac*-Res-Act transgene, which has the *ac* mC-CRE activation element (*ac*-mC-CRE-Act) cloned to the *ac*-promoter + *ac* gene partially rescues the MS mCs and causes formation of ectopic mCs.


WT
A
B
Dfsc10-1/Y
C
Dfsc10-1/Y; ac promoter-ac
D
Dfsc10-1 /Y; ac-Res1
E
Dfsc10-1 /Y; ac-Res2
F
Dfsc10-1 /Y; ac-Res3
Dfsc10-1 / Y; ac-mC-CRE
G
H
Dfsc10-1 / Y; ac-mC-CRE-Act

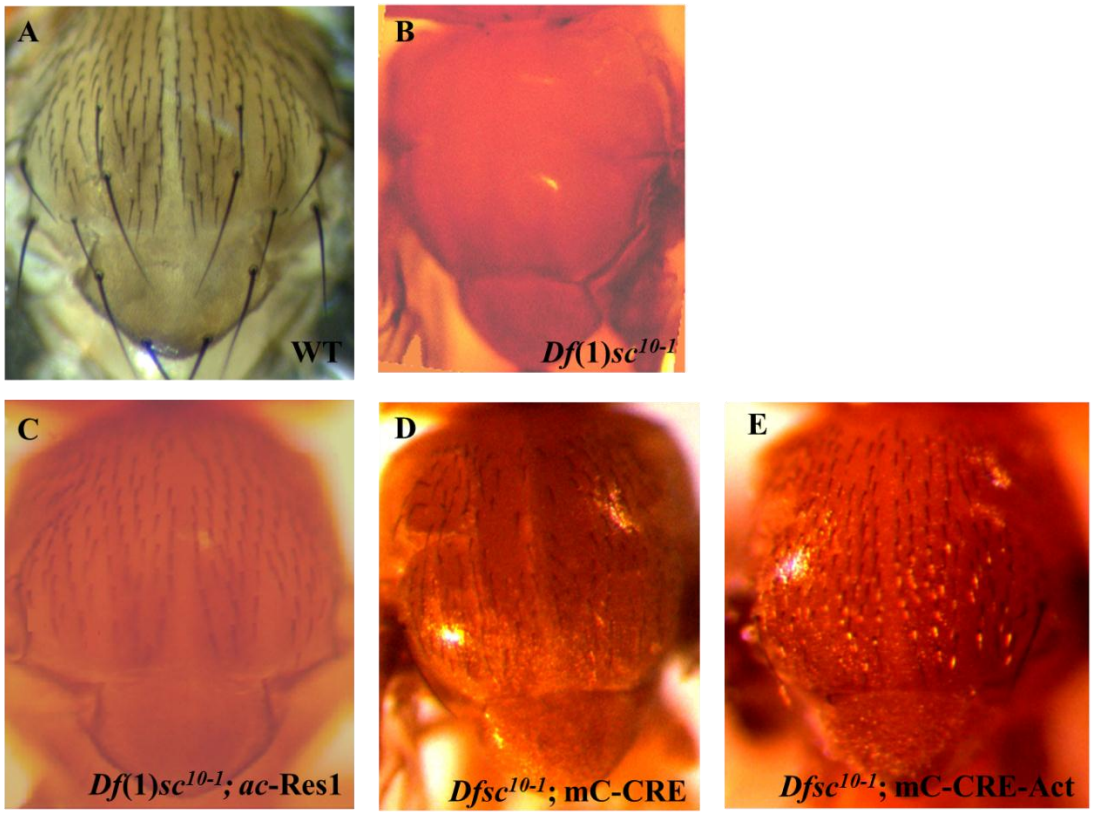


Figure 14. Phenotypes of adult nota carrying *ac* rescue constructs.

(A) Wild type notum exhibiting precisely positioned MCs and MS mCs, which are organized in longitudinal rows.

(B) An adult *Df(1)sc*$^{10\text{-}1}$ /Y notum has no sensory bristles.

(C-E) Adult *Df(1)sc*$^{10\text{-}1}$*/Y* nota carrying different transgenic rescue constructs: (C) the *ac*-Res1 transgene rescues most notal MS mC bristles. No MCs are observed. (D) The *ac*-Res-FL transgene rescues many notal MS mCs, although some bald patches are visible. (E) The *ac*-Res-Act transgene rescues most of the notal MS mCs, but not MCs.

that the *ac*-Res1 transgene contains sequences that function to control *ac* expression specifically in the primordia of late-specified sense organs.

3.3. Identification of a single *ac cis*-regulatory element that directs *ac* expression in the leg microchaete proneural fields and rescues all the leg microchaete

To further narrow down the location of a potential CRE that directs *ac* expression in the mC primordia, we tested the rescue potential of two additional transgenes, *ac*-Res2 and *ac*-Res3, made by deleting sequences from the *ac*-Res1 transgene, as shown in Fig. 11C. *ac*-Res2 and *ac*-Res3 have disruptions in the sequences near the *In(1)sc*^{v2} breakpoint (Figure 11B-C). The *ac*-Res2 fragment is missing sequences between *Hind*III and *EcoR*I restriction sites, which span the *In(1)sc*^{v2} breakpoint, and the *ac*-Res3 fragment is missing the region 3'of the *BamH*I restriction site, also spanning the *In(1)sc*^{v2} break point. The *ac*-Res2 and *ac*-Res3 transgenes confer partial rescue in the leg. The *ac*-Res2 transgene rescues fewer bristles than the *ac*-Res3 transgene, exhibiting large gaps along the leg circumference and the P/D axis. *ac*-Res3 confers better rescue along the leg circumference but also exhibits gaps along the P/D axis. Curiously, both transgenes exhibit a gradient of rescue along the P/D axis. Sense organ development appears to be preferentially rescued more proximally in the leg overall. Within each leg segment, it appears that bristles are missing from the distal ends. (Figure 13E and F).

To complement the analysis of sense organ rescue in the adult, we examined Ac expression from the *ac*-Res2 and *ac*-Res3 transgenes in a *Df(1)y*^{3PL}*sc*^{8R} background. Surprisingly, although each transgene confers partial rescue in the adult, the expression

directed by each transgene differs significantly. We find that the *ac*-Res3 transgene directs expression of Ac in the mC proneural fields of mid-prepupal legs (Figure 15E). Consistent with the adult mC phenotype, Ac expression is missing from the distal region of each segment in prepupal legs (Figure 15E). Also note that the expression of *ac* in legs from the *ac*-Res3 transgene and *In(1)sc*^{v2}/Y males are very similar, hinting that the 3' sequences deleted in the *ac*-Res3 transgene may be important for bristle formation in the distal regions of each leg segment. The *ac*-Res2 gene, however, does not confer proneural gene expression, although sporadic expression is observed in some SOPs (Fig 15C). Given that the *ac*-Res2 transgene confers no proneural gene expression in mC primordia of prepupal legs, it is surprising that it can rescue even a few sense organs in the adult. A potential explanation for this observation is that the sporadic expression in SOPs is sufficient for low level rescue of mC development in the adult.

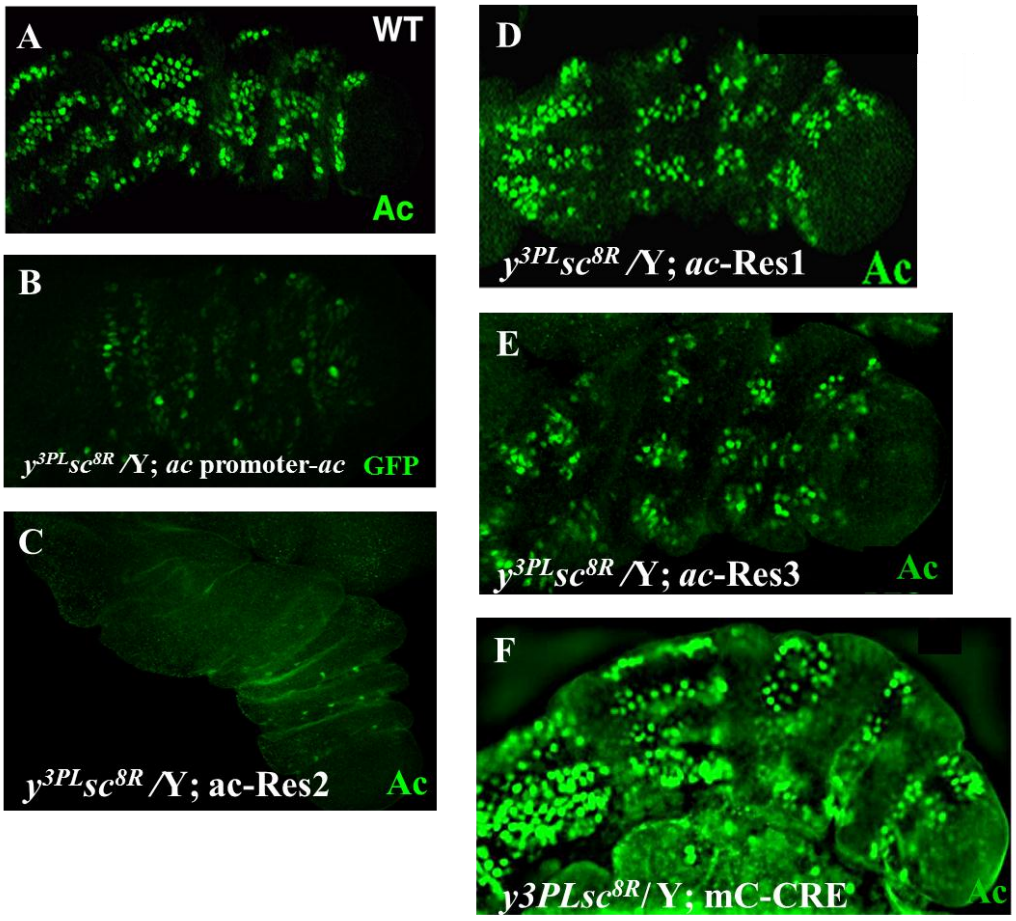
Our observations suggest that the *ac*-Res3 transgene contains an, at least partially, functional mC CRE, which can direct mC proneural expression. On the other hand, the putative mC CRE is likely disrupted in the *ac*-Res2 transgene, suggesting that sequences necessary for the mC CRE function are contained between the *Hind*III and *Bam*HI sites 3' to the *ac* coding region. Together with the analysis of the inversion mutants, this data suggests that an *ac* mC-CRE maps to the sequences between the *Hind*III site 3' to the *a*c coding region and the *Sal*I site 3' to the *In(1)sc*^{v2} breakpoint (Figure. 11B). Therefore, we cloned a 4kb *Sal*I fragment, which spans most of the region to which we have mapped the mC-CRE, into rescue and reporter constructs, as described above. The rescue and reporter transgenes made with the 4kb *Sal*I fragment will, henceforth, be referred to as the *ac*-Res-FL (full length 4kb *Sal*I fragment) and *ac*-GFP-FL transgenes, respectively (Figure 11D).

Figure 15. Ac expression in prepupal legs carrying rescue and reporter *ac* transgenes.

(A) Ac expression (anti-Ac) in a wild type mid-prepupal leg.

(B) GFP expression in a prepupal leg carrying *ac* promoter-*GFP* transgene. Note that Ac is expressed in a few single nuclei, corresponding to the SOP nuclei, but there is no proneural Ac expression.

(C-F) Expression of Ac in mid-prepupal $y^{3PL}sc^{8R}$ /Y legs carrying different transgenic rescue constructs: (C) *ac*-Res2 exhibits Ac expression in a few SOPs, but does not rescue proneural *ac* expression. (D) the *ac*-Res1 directs Ac expression pattern in a wild type pattern (E) the *ac*-Res3 transgene directs ac expression proneural stripes along the leg circumference, but expression is missing in the distal half of each tarsal segment. (F) the *ac*-Res-FL transgene directs Ac expression pattern in a wild type pattern.


A
WT
Ac
B
y3PLsc8R /Y; ac promoter-ac GFP
C
y3PLsc8R /Y; ac-Res2 Ac
D
y3PLsc8R /Y; ac-Res1 Ac
E
y3PLsc8R /Y; ac-Res3 Ac
F
y3PLsc8R/ Y; mC-CRE Ac

In *Df(1)sc*$^{10\text{-}1}$/Y males, the *ac*-Res-FL rescue transgene rescues the leg and notal mCs to a degree similar to that observed with the *ac*-Res1 transgene. Most MS mCs but no MCs or CS mCs are rescued (Figure 13G). Also, the endogenous *ac* expression pattern driven by the *ac*-Res-FL transgene in a *Df(1)y*^{3PL}*sc*^{8R} background (Figure 15F) recapitulates wild type expression (Figure 15A), as does the *ac*-Res1 transgene(Figure 15D). These findings suggest that the *ac*-Res-FL transgene contains a CRE that can direct expression in the leg and notal mCs and in conjunction with the *ac* promoter and coding sequences confers rescue of mC bristles in the adult. Therefore, we will refer to the 4kb *Sal*I fragment as the *ac* mC-CRE. Together, these results indicate that distinct mechanisms are used to establish proneural gene expression in the primordia of the mCs *vs.* that of the notal MCs. Expression of *ac* in the notal MC proneural fields is controlled by discrete, modular *cis*-regulatory elements spread out over a 100 kb region in the ASC. On the other hand, one single CRE can drive *ac* expression in all the leg and notal mC primordia. It is possible that the organization of the *ac* mC CRE is complex and consists of smaller elements that direct expression of *ac* in subsets of the overall pattern observed with the larger element. However, further analysis of the 4kb *Sal*I , described below, suggests that this is not the case.

3.4. An *ac* mC-CRE GFP reporter construct recapitulates the pattern of mC proneural fields

In order to further investigate the function of the *ac* mC-CRE, we made reporter constructs with the full-length and wild type versions of the mC-CRE, which are described in this and the following chapter. For this analysis, we used the GFP reporter vector, pStinger,

(Barolo et al., 2000) which was modified by addition of the *ac* promoter 5' to the *GFP* gene. All versions of the mC-CRE were cloned 5' to the *ac* promoter (Figure 11C and D). Even though the *ac* promoter itself cannot direct proneural expression, it is required for the function of *ac* CREs because the promoter contains several critical protein binding sites: three E-Boxes, which Ac and Daughterless (Da) heterodimers bind for self-activation of *ac* transcription (Martinez et al., 1993; Van Doren et al., 1991) and a Hairy binding site for repression (Van Doren, et al. 1994; Ohsako et al., 1994). In prepupal legs, the *ac*-GFP-FL reporter gene recapitulates endogenous *ac* expression in the leg mC primordia (Figure 16A), consistent with our observations with the *ac*-Res-FL rescue transgene.

3.5. The *a*c mC-CRE is responsive to Hairy and Delta/Notch signaling

Previously we have shown that, in the leg, the pattern of spatially refined *ac* expression is established by two prepattern regulators, Hairy and Delta/Notch signaling (Joshi et al., 2006), unlike in the notum where Dl alone spatially refines *ac* expression in the mC proneural fields (Parks et al., 1997). Therefore, we examined whether the *ac* mC-CRE-GFP reporter construct is responsive to Hairy and Notch signaling. The UAS/Gal4 system (Brand and Perrimon, 1993) was utilized to ectopically express *hairy* or to ectopically activate Dl/N signaling. For these experiments, we employed the *rotund-Gal4* (*rn-Gal4*) driver, which directs *Gal4* expression in leg tarsal segments 2-4. Expression of *UAS-hairy* driven by *rn-Gal4* results in loss of *ac* mC-CRE-GFP expression throughout tarsal segments 2-4, suggesting that mC-CRE-GFP is responsive to Hairy (Figure 16B). Secondly, we examined whether this construct is responsive to N by ectopically expressing a constitutively active

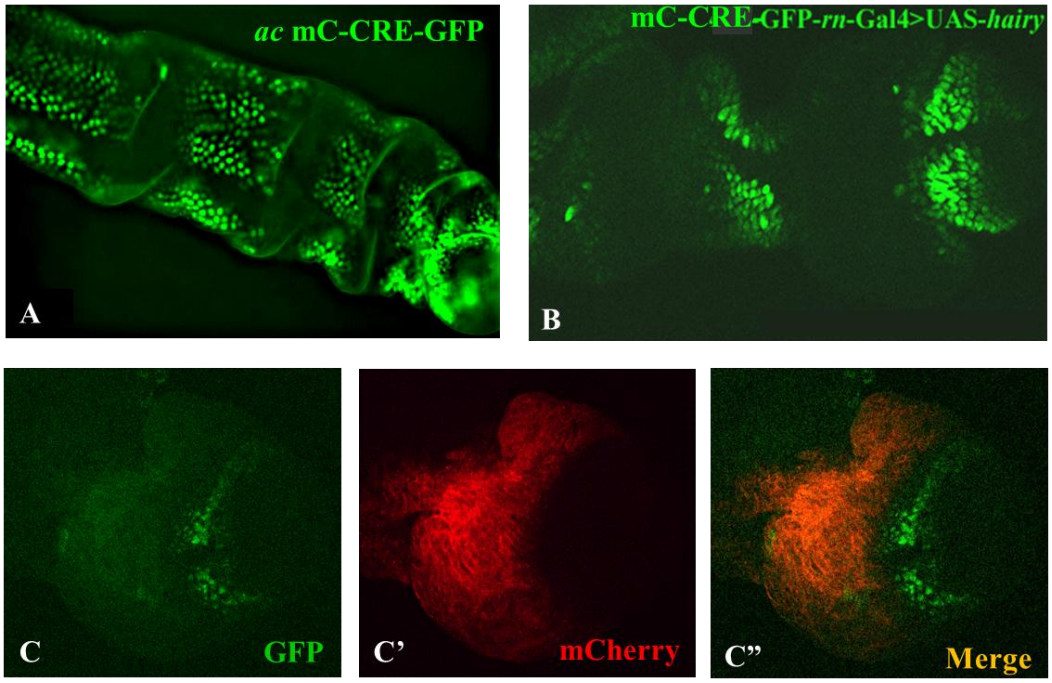


Figure 16. GFP Expression pattern and responsiveness to Hairy and Notch signaling of the *ac*-GFP-FL reporter transgene.

(A) The *ac*-GFP-FL reporter transgene (labeled *ac*-mC-CRE-GFP in panel A) recapitulates the wild type *ac* expression pattern in mid-prepupal legs.

(B) Over and ectopic expression of *hairy*, using UAS-*hairy*, under the control of *rn*-Gal4, abrogates GFP expression directed by the *ac*-GFP-FL transgene, indicating that this transgene is responsive to Hairy.

(C-C'') A *rn-Gal 4* driver was used to direct UAS-*NICD(III)* and UAS-*mCherry* in mid-prepupal legs carrying the *ac*-GFP-FL transgene. Expression of *NICD* causes constitutive activation of the Notch signaling pathway, resulting in loss of GFP expression (C, C'') directed by the *ac*-GFP-FL transgene. Cells expressing NICD are marked by mCherry expression (C', C''). This suggests that *ac*-GFP-FL transgene is responsive to N signaling.

form of the N receptor, N intracellular domain (NICD) under control of *rn-Gal4*. GFP expression was abrogated in the *rn* expression domain, suggesting that the *ac* mC-CRE-GFP construct is also responsive to Notch signaling (Figure 16C-C"). These results are consistent with our previous genetic data (Joshi et al., 2006) and suggest that the *ac* mC-CRE has Hairy and Dl/N response sequences.

3.6. Identification of an activation element of *ac* mC-CRE, *ac* mC-CRE-Act

Our analysis of *ac* expression in legs bearing breakpoint mutations and from the rescue constructs shown in Figure 11B-C, suggest that sequences between the *Hind*III and *Eco*RI sites 3' to the *ac* coding region are essential for function of the *ac* mC-CRE. Lack of these sequences in the *ac*-Res2 rescue construct abrogates proneural gene expression in the leg mC primordia. The *ac*-Res3 construct, on the other hand, directs substantial proneural expression and the *ac*-Res-FL rescue construct recapitulates the full proneural gene expression in the leg mC primordia. These data suggest that the *Sal*I/*BamH*I fragment at the 5' end of the *ac* MC-CRE contains sequences essential for the ability of the *ac* mC-CRE to direct proneural gene expression. We, therefore, made a reporter (*ac*-GFP-Act) and rescue construct (*ac*-Res-Act) with the *Sal*I/*Bam*HI fragment. The *ac*-GFP-Act reporter directs broad expression of GFP along the leg circumference, including the Dl/N-responsive *hairy-OFF* interstripes (17A-A"). However, expression is excluded from *hairy*-expressing cells, which is likely due to Hairy repression mediated by the Hairy binding site in the *ac* promoter. These observations suggest that the sequences in *ac*-GFP-Act reporter are sufficient to activate broad expression of *ac* along the leg circumference, and hence, we refer to the *Sal*I/*BamH*I fragment as the *ac*

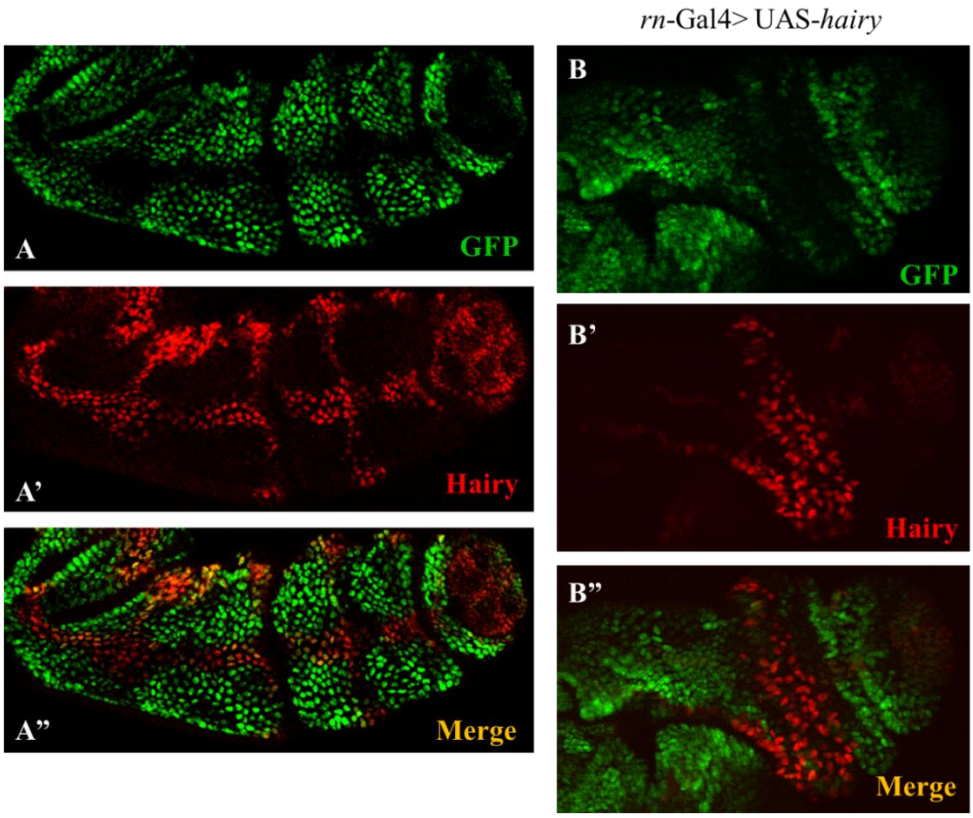


Figure 17. The *ac* mC-CRE activation element directs broad GFP expression and is responsive to Hairy.

(A) The *ac*-GFP-Act transgene directs broad GFP expression (green) along the circumference of mid-prepupal legs but is not expressed in the *hairy*-On interstripes (A, A''), Hairy expression (red in A' and A") was visualized with anti-Hairy. The lack of expression in the *hairy*-On interstripes suggests that the *ac*-GFP-Act transgene is Hairy responsive.

(B) Over and ectopic expression of *hairy*, using UAS-*hairy*, under the control of *rn*-Gal4, abrogates GFP expression directed by the the *ac*-GFP-Act transgene, indicating that this transgene is responsive to Hairy.

mC-CRE activation element (*ac mC-CRE-Act*) (Figure 11C). However, the observed expression in the *hairy*-OFF interstripes suggests that *ac mC-CRE-Act-GFP* lacks sequences that mediate responsiveness to Dl/N signaling. The *ac*-Res-Act transgene directs low-level expression (not shown) in prepupal legs, suggesting that additional sequences outside of the *Sal*I/*BamH*I fragment are required for activation of *ac* expression. Although expression levels from *ac*-Res-Act are low, we observe partial rescue of the leg mCs and ectopic mC bristles. The formation of ectopic mCs likely result from the broad, albeit low-level, expression of *ac* directed by *Sal*I/*BamH*I fragment.

3.7. *ac* mC-CRE-Act GFP reporter responds to Hairy and Dl/N signaling

The expression from the mC-CRE-Act GFP reporter (*ac*-GFP-Act) suggests that it contains response sequence for Hairy but not for Dl/N signaling, while the intact mC-CRE-GFP reporter is responsive to both. To test if mC-CRE-Act GFP is responsive to Hairy and/or Dl/N we assayed its expression in legs expressing ectopic Hairy or NICD, as described above. We observe that GFP expression is compromised in legs expressing *hairy* under control of *rn*-Gal4 (Figure 13B-B"), consistent with the observation that mC-CRE-Act GFP is not expressed in the *hairy*-ON interstripes. As mentioned, Hairy likely acts through the Hairy site in the *ac* promoter. Hairy has been shown to repress *ac* expression by binding *ac* promoter in the wing (Van Doren et al., 1994; Ohsako et al., 1994) and we report, in the next chapter, that Hairy represses *ac* expression in the prepupal leg as well through the Hairy site in the *ac* promoter.

The results of ectopic activation of N signaling are less clear as contradictory results are obtained with two independent UAS-NICD lines, one which is inserted on the X-chromosome [*UAS-NICD (X)*] and the other on the 3rd chromosome [*UAS-NICD(III)*]. With both lines, expression of *UAS-NICD* under control of *rn-Gal4* results in overgrowth of the prepupal legs (Fig. 18). However, *UAS-NICD(III)* causes significant reduction of *mC-CRE-Act-GFP* expression (Figure 18B-B"), while *UAS-Nintra(X)* does not appear to have an effect. The different results observed with the two insertions could be explained by the relative levels of expression from each line. While both lines can cause overgrowth of legs discs, the levels of *NICD* may not be sufficient to inhibit *mC-CRE-Act-GFP* expression with *UAS-NICD(X).* The loss of *GFP* expression in response to *UAS-NICD(III)* is not consistent with the expression of the *mC-CRE-Act-GFP* in the *hairy*-OFF stripes. This would suggest that physiological levels of N signaling are not sufficient to inhibit *mC-CRE-Act-GFP* expression. However, expression of *UAS-Nintra(III)* under *rn*-Gal4 control may result in super-physiological levels or N signaling, which can inhibit *mC-CRE-Act-GFP* expression. It is plausible that N signals through the Hairy response sequence in the *ac* promoter, which is similar to an E(spl) response site. Alternatively, it is plausible that N signals through a sequence in the mC-CRE. These possibilities are further explored in the next chapter.

3.8. Discussion

Proper spatial regulation of proneural genes is important for periodic longitudinal rows of mechanosensory microchaete in the *Drosophila* leg. Genetic studies in our laboratory have shown that Delta/Notch signaling, together with the bHLH transcriptional repressor Hairy,

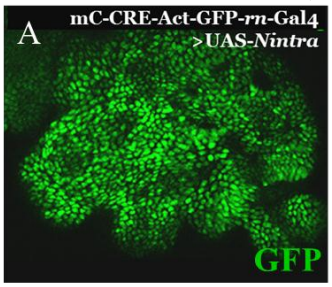
Figure 18. The *ac*-GFP-Act transgene is responsive to Notch signaling.

Two UAS-*NICD* transgene insertions, one on the X chromosome, UAS-*NICD*(X) and another on the 3rd chromosome, UAS-*NICD*(III) were used to assay the effect of constitutive Notch signaling on *ac*-GFP-Act expression.

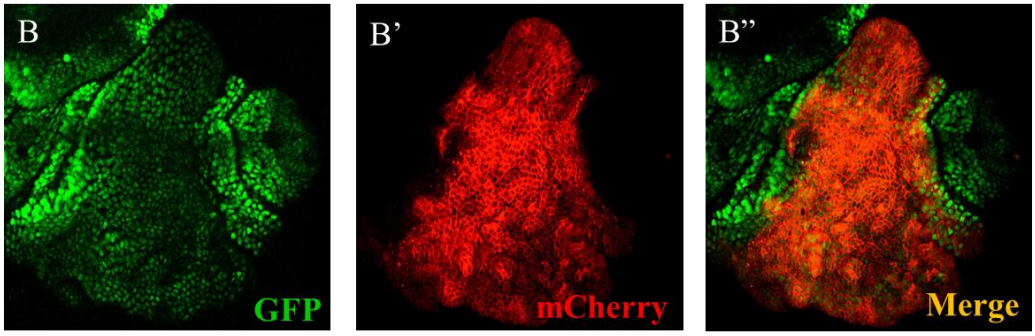
(A) A *rn-Gal 4* driver was used to direct UAS-*NICD(X)* in mid-prepupal legs carrying the *ac*-GFP-Act transgene. This causes overgrowth of the leg in the *rn-Gal4* expression domain but does not compromise *ac*-GFP-FL directed expression, suggesting that *ac*-GFP-Act is not responsive to Notch signaling.

(B-B") On the the other hand, when the *rn-Gal 4* driver was used to direct UAS-*NICD(III)* and UAS-*mCherry* (B', B'') in mid-prepupal legs carrying the *ac*-GFP-Act transgene, GFP expression was lost (B, B"). This suggests that *ac*-GFP-Act transgene is responsive to Notch signaling. A potential explanation for the discrepant results is provided in the text.

rn-Gal4> UAS-*NICD(X)*



rn-Gal4> UAS-*mCherry*, UAS-*NICD(III)*



establish periodic *ac* expression in the mC proneural fields (Joshi et al., 2006). Studies on the mechanosensory macrochaete (MS MC) patterning in the notum have shown that there are several CREs, scattered throughout the *ac-sc* complex, each responsive to local prepattern transcription factors, which regulate proneural gene expression in particular proneural clusters. However, we have identified a single *cis*-regulatory enhancer element, *ac* mC-CRE, that can direct *ac* expression in all the mC primordia in the leg and the notum.

We generated transgenic fly lines carrying rescue and GFP reporter constructs. The *ac* mC-CRE rescues almost all the mC, but not macrochaete, in flies that lack functional Ac and Sc proteins, indicating this element is specific for the late specified sensory organs. The expression patterns of *ac*, in the prepupal leg, which lacks functional Ac protein, and GFP recapitulates narrow mC proneural fields as observed in wild type prepupal legs. This enhancer, *ac* mC-CRE, has an activation element that activates *ac* expression broadly along the leg circumference in the absence of repressive cues. As discussed, the *ac* promoter contains a Hairy response sequence, suggesting that repressive cues may go through this element. However, the relative expression patterns from the *mC-CRE-Act-GFP* and *mC-CRE-GFP* transgenes, implies that sequences in the 3' *Bam*HI/*Sal*I region contain additional elements that are responsive to repressors. These results have allowed us to outline a model for patterning of mC; Expression of *ac* in the mC proneural fields can be broadly activated by not yet identified activators, that act through the activation element of *ac*-mC-CRE, but spatially refined by repression mediated by two prepattern regulators, Hairy and Notch signaling, which are established earlier, through the repression element of *ac* mC-CRE and the Hairy binding site in *ac* promoter.

IV. MOLECULAR AND FUNCTIONAL ANALYSIS OF A HAIRY AND DELTA/NOTCH RESPONSIVE *cis*-REGULATORY ELEMENT THAT CONTROLS *achaete* EXPRESSION IN THE MICROCHAETE PRONEURAL FIELDS

4.1. Introduction

The Drosophila adult PNS is an ideal model system to investigate the genetic and molecular mechanisms that control the formation of complex morphologies. The surface of the Drosophila adult cuticle is studded with myriad external sensory organs that are organized in stereotypic patterns. Previous studies have shown that development of complex sense organ arrangements is contingent on prior expression of the proneural genes, *ac* and *sc*, within the limb and body wall primordia in patterns that prefigure the stereotyped sense organ arrays observed in the adult. This implies that regulation of spatially defined proneural gene expression is a prerequisite for proper development of the adult morphology.

Most studies have focused on the development of early-specified sensory organs, specifically, the MCs found on the notum. There are eleven MCs on each half of the notum, or heminotum, arranged in a mirror-image pattern. Extensive genetic and molecular studies have shown that patterning of the notal MCs requires expression of *ac*/*sc* in proneural clusters at defined positions of the notal primordia. Expression of *ac/sc* expression in the MC proneural clusters is controlled by locally expressed activators and repressors. These regulators of *ac/sc* expression act through several modular CREs, each of which controls *ac/sc* expression in a discrete proneural field.

Our genetic and preliminary molecular studies, on the other hand, suggest a distinct mechanism is used to establish *ac/sc* gene expression in the proneural fields of the late-specified and more numerous mCs. Based on these studies, we proposed the model shown in Figure 10, which suggests that expression of *ac/sc* is activated simultaneously in all the mC proneural fields of the leg and notal imaginal discs by a uniformly expressed activator(s). Periodic expression is established by locally acting repressors, which are expressed in spatially defined patterns. Preliminary analysis of a CRE, the *ac-mC-CRE*, that controls *ac* expression in leg and notal mC proneural fields suggests that activation of *ac/sc* expression is mediated by a single activation element, rather than separate elements, such as those that activate expression locally in the MC primordia of the notal disc. Another aspect of this regulation is that expression in the mC proneural fields is not activated until mid-prepupal leg development. Premature activity of the *ac*-mC-CRE would likely disrupt the patterning of the early-specified sense organs.

To test this model and gain insight into the molecular mechanisms that control patterning of late-specified sense organs, we have investigated the function of the *ac*-mC-CRE. Molecular analysis of this CRE should provide important insights into several outstanding questions regarding the mechanisms involved in patterning of mC bristles. For instance, is the expression of *ac* in the mC proneural fields controlled by a uniformly expressed activator(s) or by combined function of several locally acting factors? Does the *ac*-mC-CRE contain sequences that mediate temporal control of *ac* expression? Do common sequences in the *ac*-mC-CRE mediate response to bHLH repressor, Hairy, and the E(spl) bHLH repressors that mediate repression by Dl/N or are there distinct response sites?

This chapter focuses primarily on how the *ac*-mC-CRE responds to repressive inputs from Hairy and Dl/N signaling. This is a critical aspect of mC bristle patterning because our model predicts that the complexity of *ac* expression pattern in the mC proneural fields is established largely by prior expression of repressors in spatially defined patterns. This raises the question of how patterning information from this repressive prepattern is interpreted by the *ac*-mC-CRE. How much complexity is there in the *ac*-mC-CRE in terms of response to Hairy and Dl/N signaling? Knowledge of the mechanisms of *ac*-mC-CRE function will provide insight not only into sense organ patterning but also how morphological novelty is generated. For example, altered function of repressor response sites in the *ac*-mC-CRE would likely have very different effects on sense organ morphology depending on whether common vs. distinct sites mediate response to Hairy and Dl/N signaling.

4.2. Activation of *achaete* expression in the leg and notal mC proneural fields

Our model for development of late-specified sense organs of the leg and notum suggests that *ac/sc* expression is activated broadly and in all the mC proneural fields by a uniformly expressed activator(s). Preliminary analysis of the *ac*-mC-CRE, which controls *ac/sc* expression the mC proneural fields, suggests that activation of *ac/sc* expression is mediated by a single activation element contained within this CRE. This activation element, *ac*-mC-CRE-act, which is contained within a 600 bp *Sal*I + *Bam*HI fragment at the 5' of the *ac*-mC-CRE, was tested in the rescue (*ac*-Res-Act) reporter and GFP reporter (*ac*-GFP-Act) vectors described in Chapter 3. In these vectors, the *ac* gene and the GFP reporter gene are under control of the *ac* promoter (Figure 11C and 11D). We observe that the *ac*-GFP-Act

directs expanded expression of GFP relative to the *ac*-GFP-FL. Although the *ac*-mC-CRE-Act does not appear to have potential Hairy and Dl/N response sequences, GFP expression is excluded from the *hairy-On* interstripes, while it overlaps the *hairy*-Off interstripes (Figure 17A-A"), suggesting that Hairy-responsiveness is likely mediated by a previously identified Hairy binding site in the *ac* promoter (Ohsako et al., 1994; Van Doren et al., 1994).

Our model predicts that in the absence of repressive inputs from Hairy and Dl/N signaling, *ac/sc* expression would be activated uniformly along the leg circumference. To test this prediction, the Hairy site in the *ac* promoter was mutated in the *ac*-*GFP*-Act reporter construct (*ac*-GFP-Act-H1*). In agreement with our prediction, we observe that this results in uniform GFP expression along the leg circumference (Figure 19C-C"). This finding also indicates that Hairy acts through the Hairy-binding site in the *ac* promoter and not through the *ac*-mC-CRE activation element. Although, the uniform expression directed by *ac*-mC-CRE-act is consistent with our suggestion that *ac/sc* expression is induced by a ubiquitously expressed transcriptional activator(s), we have not ruled out the possibility that expression is controlled by multiple locally-acting factors that act through this element.

4.3. Hairy-mediated repression of *achaete* expression during mC specification

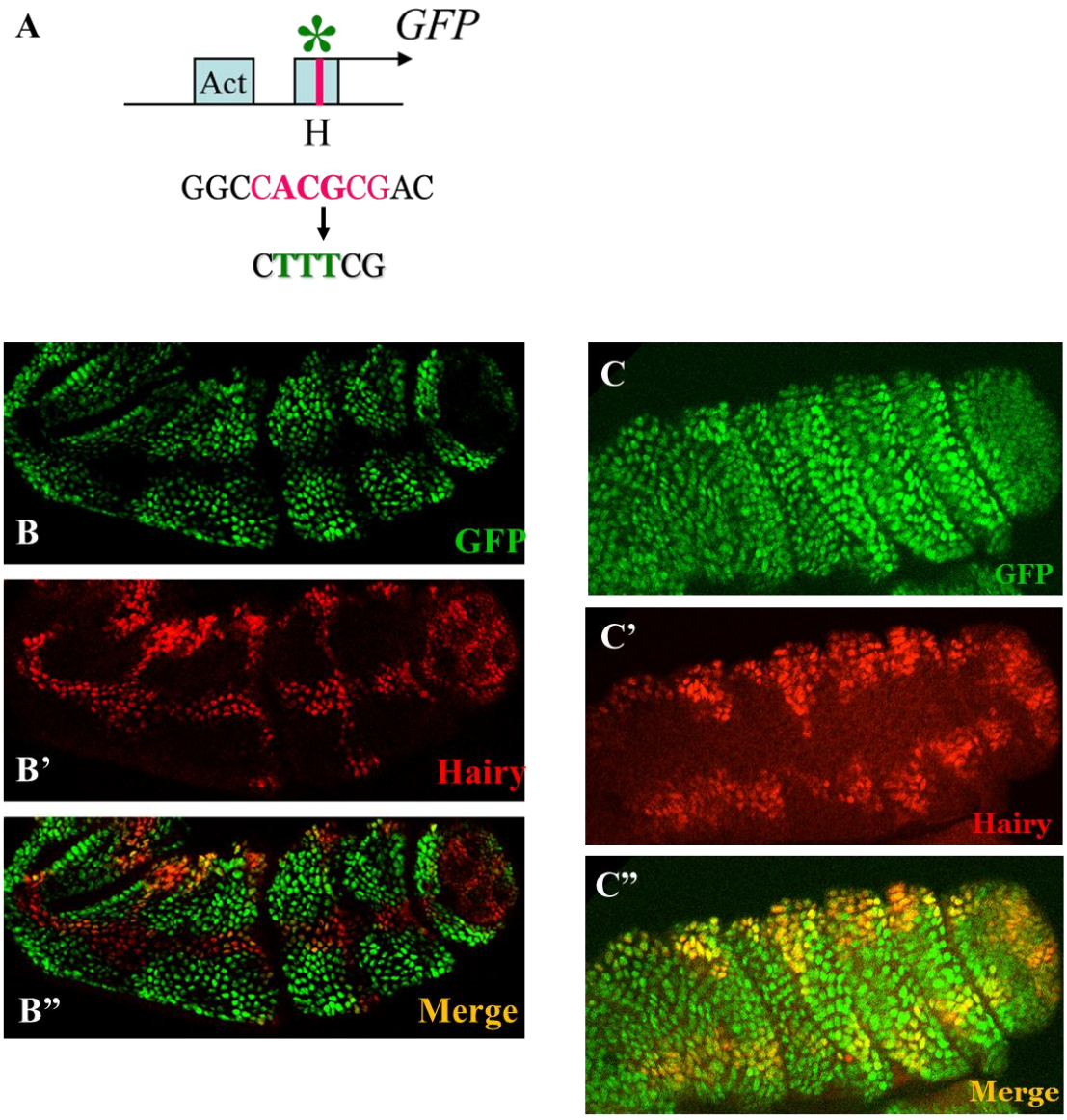
Hairy, a bHLH transcription repressor is a prepattern regulator of proneural *ac*/*sc* expression. In the absence of *hairy* function, mC sensory organs are disorganized in the adult legs and ectopic bristles are observed on the wing due to expanded expression of *ac* (Ingham et al., 1985; Orenic et al., 1993). Here, we ask if response to Hairy is mediated mainly by the Hairy site in the *ac* promoter, or if there are additional response sequences in the *ac*-mC-CRE.

Figure 19. The *ac*-mC-CRE activation element can direct uniform *ac* expression in prepupal legs when function of the Hairy binding sequence in the *ac* promoter is compromised.

(A) Schematic map of the *ac*-GFP-Act-H1* transgene. This reporter construct was generated by introducing a mutation in the Hairy binding sequence (see text) in the *ac* promoter (H1) of the *ac*-GFP-Act transgene (Figure 11). The asterisk designates that the H1 site has been mutated.

(B-B") The *ac*-GFP-Act transgene directs broad GFP expression (green in B, B''), but expression is excluded from Hairy-expressing cells (red in B', B''), suggesting that the transgene is Hairy-responsive.

(C-C") The *ac*-GFP-Act-H1* directs uniform GFP (green in C, C'') in a prepupal leg, overlapping Hairy expression (anti-Hairy, red in C', C''). This observation indicates that the *ac*-mC-CRE activation element can direct uniform expression of ac in the absence of repressive inputs. In addition, this finding suggests that the *ac* promoter H1 site mediates response to Hairy in prepupal legs.


A
GFP
Act
H
GGCCACGCGAC
CTTTCG
B
GFP
B'
Hairy
B"
Merge
C
GFP
C'
Hairy
C"
Merge

4.3.1. Periodic *achaete* expression is mediated by a Hairy-response sequence in the *achaete* promoter.

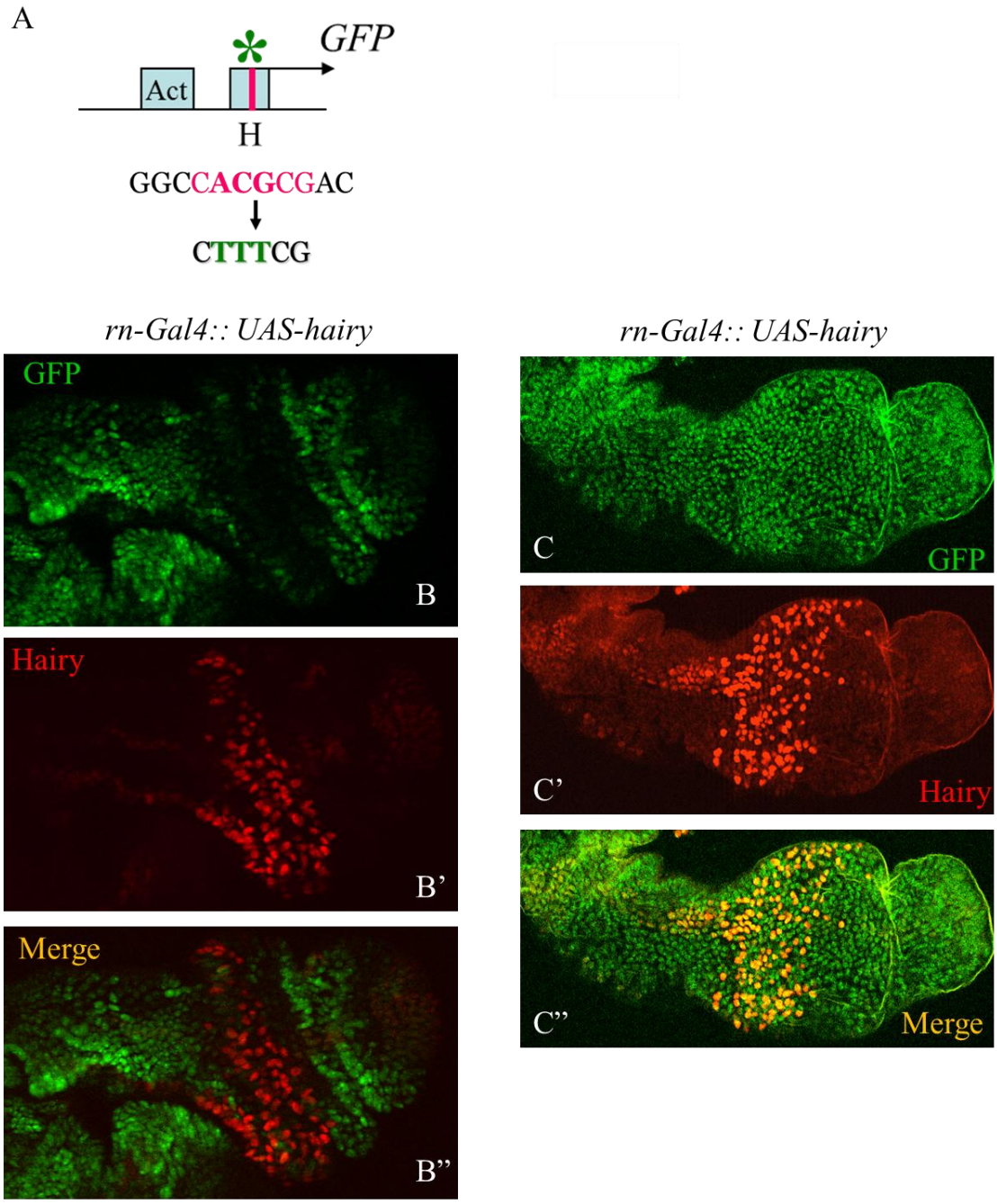
As mentioned in the previous section, the *ac*-GFP-Act reporter transgene directs expression of GFP all along the leg circumference except in Hairy expressing cells, suggesting that it is responsive to Hairy. Hairy has previously been reported to bind a sequence, **CACGCG**, called a C-box (Ohsako et al., 1994; Van Doren et al., 1994). The C-box comprises the core of a Hairy binding site in the *ac* promoter, GGC**CACGCG**AG (core Hairy binding nucleotides are in bold), that has been shown to mediate Hairy repression of *ac* in the wing imaginal disc (Ohsako et al., 1994; Van Doren et al., 1994), but has not been demonstrated to function in regulation of *ac* expression in leg discs. To determine if this site mediates response to Hairy in the leg, the C-box core in the GGC**CACGCG**AG sequence was mutated to GGC**CTTTCG**AG (sequences that were altered are underlined; Figure 19A). The mutations were made in both the *ac*-GFP-Act reporter (*ac*-GFP-Act-H1*) and rescue (*ac*-Res-Act-H1*) constructs. We find that expression from the *ac*-GFP-Act-H1* reporter is expanded into the Hairy-expressing interstripe domains, such that GFP is uniformly expressed throughout the prepupal leg (Figure 19C-C"). This suggests that Hairy acts through this sequence to repress *ac* expression in prepupal legs, as well as in the wing disc. Consistent with the observations with the reporter gene, *ac*-Res-Act-H1* directs uniform expression of *ac* along the leg circumference (Figure 21B-B"). Surprisingly, however, along the P/D axis, *ac* expression is excluded from cells near the joints, while the reporter transgene directs uniform GFP expression along the P/D axis. Note in Figure 21B-B" that *ac* expression from the rescue construct overlaps Hairy expression, which is never observed with the wild type rescue transgene (Figure 15F). This indicates that Hairy responsiveness

Figure 20. The *ac* promoter Hairy binding site mediates responsiveness to Hairy in prepupal legs.

(A) Schematic map of the *ac*-GFP-Act-H1* transgene. This reporter construct was generated by introducing a mutation in the Hairy binding sequence (see text) in the *ac* promoter (H1) of the *ac*-GFP-Act transgene (Figure 11). The asterisk designates that the H1 site has been mutated.

(B-B") Over and ectopic expression of Hairy using UAS-*hairy* (anti-Hairy, red in B'. B'') under control of *rn*-Gal4 results in loss of GFP expression directed by the *ac*-GFP-Act (green in B, B'') transgene, indicating that the *ac*-GFP-Act is responsive to Hairy mediated repression.

(C-C") Ectopic expression of UAS-*hairy* under control of *rn-Gal4* (anti-Hairy, red in C', C''), does not compromise GFP expression directed by the *ac*-GFP-Act-H1* reporter transgene (green, C, C''). This indicates that the H1 in the *ac* promoter mediates response to Hairy in mid-prepupal legs.


A
GFP
Act
H
GGCCACGCGAC
CTTTCG
rn-Gal4:: UAS-hairy
GFP
B
Hairy
B'
Merge
B''
rn-Gal4:: UAS-hairy
C
GFP
C'
Hairy
C''
Merge

has been compromised. In addition, since the reporter gene expression is uniform along the P/D axis, this suggests that post-transcriptional regulation of *ac* expression might underlie the lack of *ac* expression near the joints.

To confirm that mutation of the Hairy binding sequence compromises response to Hairy, we drove *UAS-hairy* expression under control of *rn-Gal4* and examined expression of *ac*-GFP-Act-H1*. While *ac*-GFP-Act expression is lost in cells ectopically expressing Hairy (Figure 20B-B"), *ac*-GFP-Act-H1* is unaffected (Figure 20C-C").

We also asked whether compromised function of the *ac* promoter Hairy-binding sequence in the *ac*-Res-Act-H1* affects the mC pattern in adult legs and wings. As discussed in Chapter 3, *ac*-Res-Act-H1* expresses *ac* at low levels but, yet, confers substantial rescue of mC bristles in $Dfsc^{10\text{-}1}/Y$ adult legs, and ectopic mCs are observed between the L-rows. On the other hand many more mCs, both in the L-rows and ectopic, are produced in legs from $Dfsc^{10\text{-}1}/Y$ animals carrying the *ac*-Res-Act-H1* transgene (Figure 21C). Similarly, in the notum, the *ac*-Res-Act-H1* (Figure 21I) transgene induces formation of more mCs than the *ac*-Res-Act transgene (Figure 14E) in a $Dfsc^{10\text{-}1}$ background. Note the disorganized L-rows on the notum from the fly carrying the *ac*-Res-Act-H1* vs the *ac*-Res-Act insertion (compare Figure 21I to Figure 14E).

Further evidence that the Hairy response has been abrogated by mutation of the Hairy binding sequence in the *ac* promoter is provided by analysis of adult wing phenotypes. The *hairy* phenotype was originally observed in the wing. In wild type flies, mC bristles are only seen along the anterior wing margin (Figure 21E). Viable hypomorphic mutations of *hairy* result in ectopic mC bristles along and between the wing veins. We observe that in both $Dfsc^{10\text{-}1}$ and wild type backgrounds, the *ac*-Res-Act-H1* transgene causes disrupted wing

Figure 21. Compromised function of the Hairy site in the *ac*-GFP-Act-H1* rescue transgene results in ectopic *ac* expression in leg and wing discs and ectopic mCs in adults.

(A) Schematic map of the *ac*-GFP-Act-H1* transgene. This reporter construct was generated by introducing a mutation in the Hairy binding sequence (see text) in the *ac* promoter (H1) of the *ac*-GFP-Act transgene (Figure 11). The asterisk designates that the H1 site has been mutated.

(B-B") Mutation of Hairy site in the *ac*-Res-Act-H1* transgene results in ectopic *ac* expression in prepupal wing discs and uniform expression of *ac* (anti-Ac, green in B, B'') along the circumference of prepupal legs. Ac expression in prepupal legs completely overlaps the *hairy*-On and *hairy*-Off interstripes (anti-Hairy, red in B', B'').

(C) Leg from $Dfsc^{10\text{-}1}$/Y adult, carrying the *ac*-Res-Act-H1* transgene exhibits rescue of mCs development and ectopic bristles between the L-rows.

(D) Ac (anti-Ac, green) is ectopically expressed throughout a wild type prepupal wing carrying the *ac*-Res-Act-H1* transgene.

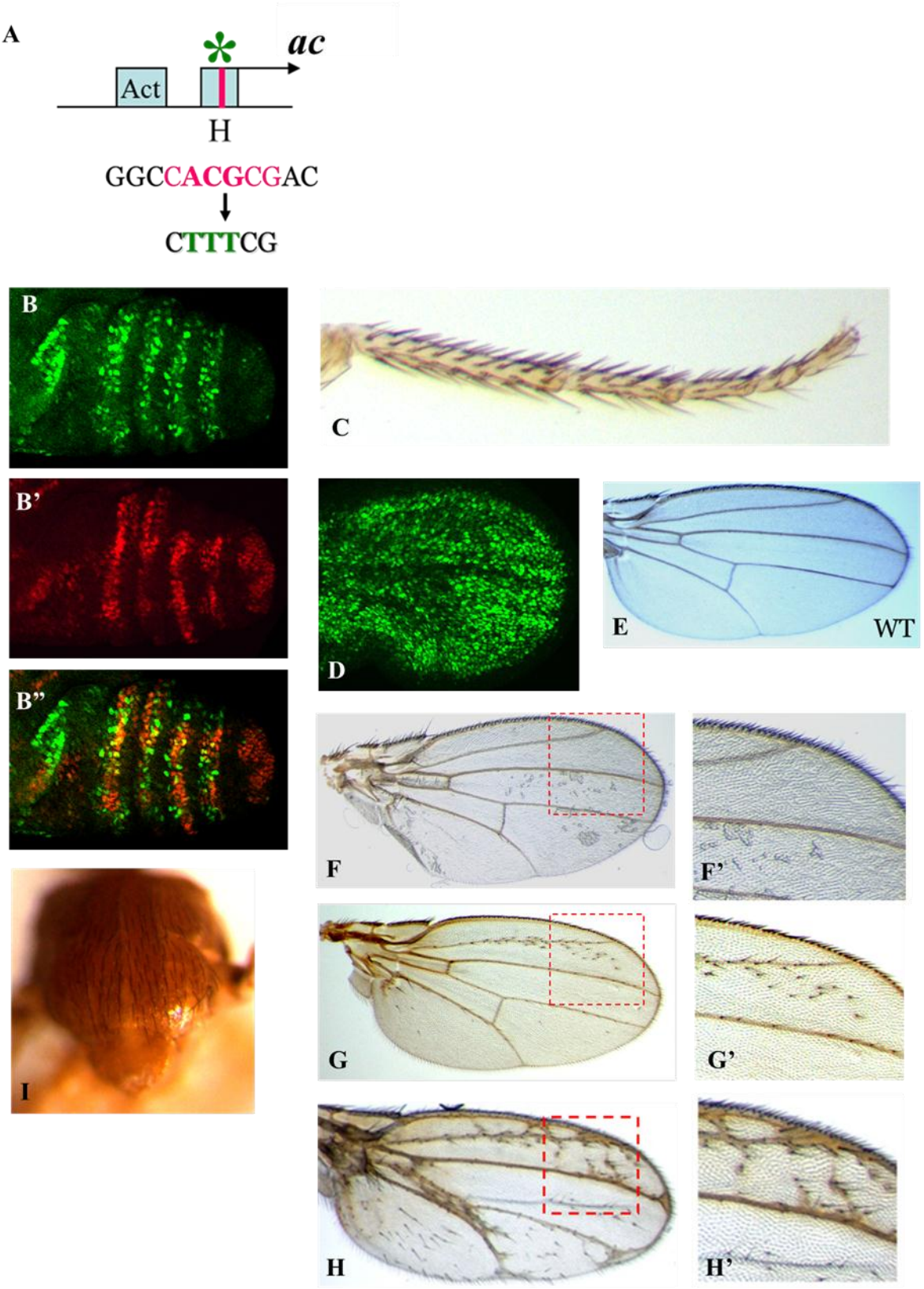
(E) Wild type adult wings exhibit mC sensory bristles only along the anterior wing margin.

(F-F') An adult wild type wing carrying the *ac*-Res-Act transgene, has no ectopic bristles. The portion of the wing, marked with a dotted red box, is magnified in panel F'.

(G-G') An adult $Dfsc^{10\text{-}1}$/Y wing carrying the *ac*-Res-Act-H1* transgene, exhibits ectopic bristles in the wing blade, most notably along the veins. The portion of the wing, marked with a dotted red box, is magnified in panel G'.

(H-H') An adult wild type wing carrying the *ac*-Res-Act-H1* transgene exhibits numerous ectopic bristles throughout the wing. The portion of the wing, marked with a dotted red box, is magnified in pane H'.

(I) An adult $Dfsc^{10\text{-}1}$/Y notum carrying the *ac*-Res-Act-H1* transgene shows rescue of most of the mCs and disorganized mC patterning.


A
ac
Act
H
GGCCACGCGAC
CTTTCG
B
B'
B"
C
D
E
WT
F
F'
G
G'
H
H'
I

vein phenotypes and formation of many ectopic bristles throughout the wing (Figure 21G-H'). In addition, *ac* is ectopically expressed throughout the blade of prepupal wings (Figure 21D). The *ac*-Res-Act transgene shows little expression in prepupal wings and exhibits no phenotype in the adult (21F-F'). The leg, notal and wing phenotypes in flies carrying *ac*-Res-Act-H1* are likely due to expanded *ac* expression, as a result of compromised Hairy-mediated repression.

4.3.2. The *ac* mC-CRE is responsive to Hairy-mediated repression

Our results presented thus far strongly support the hypothesis that Hairy functions directly through a binding site in the *ac* promoter to repress *ac* expression. These observations raise the question of whether all Hairy-mediated repression goes through the Hairy site in the *ac* promoter or if Hairy also acts through one or more sites in the *ac*-mC-CRE. In order to begin to address this question, we generated additional rescue and reporter transgenes. The transgenes described in section 4.3.1 contained the activation element from the *ac*-mC-CRE in combination with the *ac*-promoter, which was either wild type or which had a mutation in the Hairy binding site. To determine whether the 3.4 kb region that is 3' to the activation element in the *ac* mC-CRE can mediate response to Hairy-mediated repression, the full-length *ac* mC-CRE sequence was combined with the *ac* promoter with a nonfunctional Hairy binding site (Figure 22A). Both reporter (*ac*-GFP-FL-H1*) and rescue (*ac*-Res-FL-H1*) transgenes were generated. The *ac-GFP*-FL-H1* transgene drives broad GFP expression along the prepupal leg circumference, but not in the *hairy*-On interstripes (Figure 22B-B"), implying that this transgene is Hairy responsive. Recall that the *ac*-GFP-Ac-H1*, which lacks sequences 3' to the activation element, was compromised in its

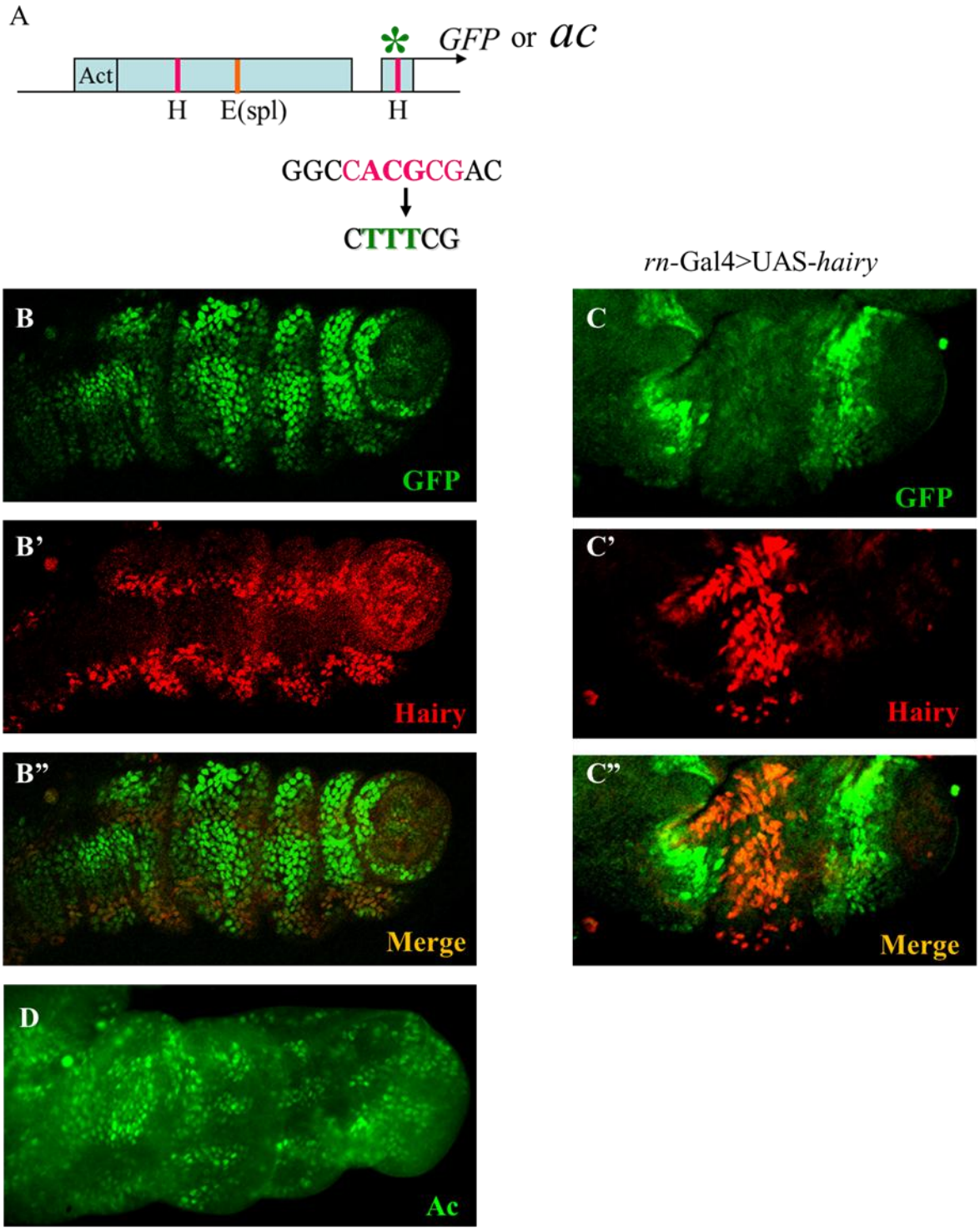
Figure 22. The Hairy site in the *ac* promoter is not essential for Hairy-mediated repression of *ac* mC-CRE activity.

(A) Schematic map of the *ac*-GFP-FL-H1* transgene. This reporter construct was generated by introducing a mutation in the Hairy binding sequence (see text) in the *ac* promoter (H1) of the *ac*-GFP-FL transgene (Figure 11). The asterisk designates that the H1 site has been mutated.

(B-B") The *ac*-GFP-FL-H1* transgene directs GFP expression (green in B, B'') that is slightly expanded into the *hairy*-Off interstripes, but not into the *hairy*-On interstripes (anti-Hairy, red in B', B'').

(C-C") Ectopic and overexpression of UAS-*hairy* under control of *rn*-Gal4 results in greatly reduced *ac*-GFP-FL-H1* directed GFP expression (green in C,C'') in Hairy expressing cells (red in C',C''). This indicates that the *ac*-GFP-FL-H1* transgene (shown in A) is Hairy responsive and that there are likely additional Hairy response sites in the *ac*-mC-CRE.

(D) $y^{3PL}sc^{8R}$/Y prepupal leg carrying the *ac*-Res-FL-H1* transgene directs Ac expression in narrow stripes (anti-Ac, in green) as observed in wild type prepupal legs. This indicates that the *ac*-REs-FL-H1* transgene (shown in A) is Hairy responsive and that there are likely additional Hairy response sites in the *ac*-mC-CRE.


A
GFP or ac
Act
H
E(spl)
H
GGCCACGCGAC
CTTTCG
rn-Gal4>UAS-hairy
B
GFP
B'
Hairy
B"
Merge
D
Ac
C
GFP
C'
Hairy
C"
Merge

response to Hairy (19C-C"). This implies that these 3' sequences have additional Hairy response sites and hence we refer to the region as the repression element.

We examined Hairy responsiveness of the *ac*-GFP-FL-H1* transgene by ectopically expressing UAS-*hairy* under control of *rn-Gal4*. GFP expression was greatly reduced in the *rn-Gal4* expression domain (Figure 22C-C"), suggesting again that this transgene is Hairy responsive. Furthermore, the *ac*-Res-FL-H1* transgene directs *ac* expression in narrow proneural stripes (Figure 22D), as observed with the *ac*-Res-FL transgene (Figure 15F). In addition, the legs, nota and wings from $Dfsc^{10\text{-}1}$/Y flies, carrying the *ac*-Res-FL-H1* transgene, exhibited wild type mC patterns.

Collectively, these results indicate that there are Hairy response sites within the *ac* mC-CRE repression element. Therefore, as described in the next section, we sought to identify potential Hairy-response sites in this element.

4.3.3. A putative Hairy-binding site in the *achaete* mC-CRE is Hairy responsive

bHLH transcription factors, such as Ac and Hairy, have been shown to bind an E-box (CANNTG) sequence (reviewed in Fisher and Caudy, 1998). In addition, bHLH repressors are known to bind either a C-box (CACNNG) or a related sequence, called an N-box (CACNAG). As discussed earlier, Hairy preferentially binds to a C-box sequence, GGC**CACGCG**AC in the *ac* promoter (Van Doren et al., 1994; Ohsako et al., 1994). We, therefore, searched the repression element for C- and N-boxes. Four potential C- or N-boxes were identified. As shown in Figure 24, two potential N-boxes are contained within the sequence: TCC<u>CTTGTG</u>**CACCAG**GC. The first sequence, CTTGTG (underlined, CACAAG on the complementary strand), is both an N and C-box and matches the Hairy

binding sequence (GGC**CACGCG**AC) in the *ac* promoter in 6/11 nucleotides; the second sequence, CACCAG, is only an N-box. A third sequence, TTG**CACGTG**GCC, has a C/E-box core and matches a consensus E(spl) binding site in 9/12 nucleotides. This sequence will be discussed in the following section. A fourth sequence, GCG**CACGCG**CA has a C-box core and matches the *ac* promoter Hairy binding sequence in 7/11 nucleotides. Of the four sequences, only the fourth is conserved (Figure 23). Because of its perfect match to the C-Box core, the close match to the *ac* promoter Hairy binding site, and its conservation in 11 other Drosophila species, we opted to assay this sequence to determine if it is required for response to Hairy. This sequence will be referred to as the Hairy-2 binding sequence (H2).

The putative Hairy-binding site, H2, was mutated (CACGCG to CTTTCG) in both rescue, *ac*-Res-FL-H1*H2*, and reporter, *ac*-GFP-FL-H1*H2*, transgenes. (Figure 26A and 25A). As shown in Figure 25B-B", the *ac*-GFP-FL-H1*H2* reporter is partially compromised in its response to Hairy. Note that, in comparison to *ac*-GFP-FL-H1* (Figure 22B-B"), there is substantial overlap between Hairy and *ac*-GFP-FL-H1*H2* expression. Correspondingly, *ac* expression from the *ac*-Res-FL-H1*H2* transgene is also expanded (Figure 26B).

The expansion of expression into the *hairy*-On interstripes observed with the *ac*-Res-FL-H1*H2* and *ac-GFP*-FL-H1*H2* transgenes suggests that mutation of both the H1 and H2 sites partly impairs response to Hairy. However, when we over- and ectopically express UAS-*hairy* under control of *rn-Gal4*, it results in loss of GFP from the *ac*-GFP-FL-H1*H2* transgenes (Figure 25C-C"; compare to *ac*-GFP-Act-H1* expression in Figure 22C-C"). These results suggest either that the mutations in the H1 and H2 sites did not completely abolish function of the H1 and H2 sites or that Hairy can repress expression through other

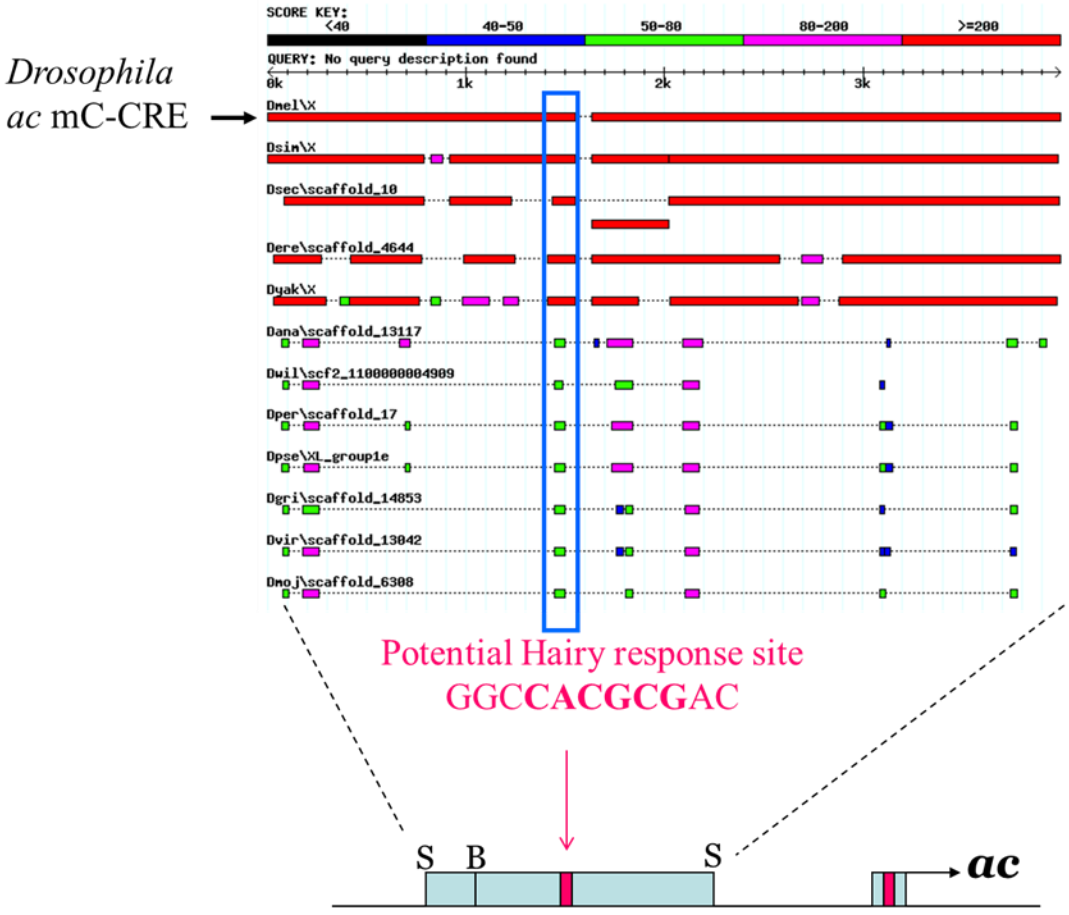


Figure 23. A putative Hairy binding site in the *ac*-mC-CRE (H2) is highly conserved.

The *Drosophila melanogaster ac*-mC-CRE sequence was compared against 11 other Drosophila species.. A potential Hairy response site sequence GGCCACGCGAC (C-box core sequence CACGCG) is well conserved in all compared Drosophila species. From top to bottom, *D.melanogaster* (*D.mel*), *D. simulans* (*D. sim*), *D. sechellia* (*D. sec*), *D. erecta* (*D.ere*), *D. yakuba* (*D. yak*), *D. ananassae* (*D. ana*), *D. willistoni* (*D. wil*), *D. persimilis* (*D. per*), *D. pseudoobscura* (*D. pse*), *D. grimshawi* (*D. gri*), *D. virilis* (D.vir), *D. mojavensis* (*D. moj*).

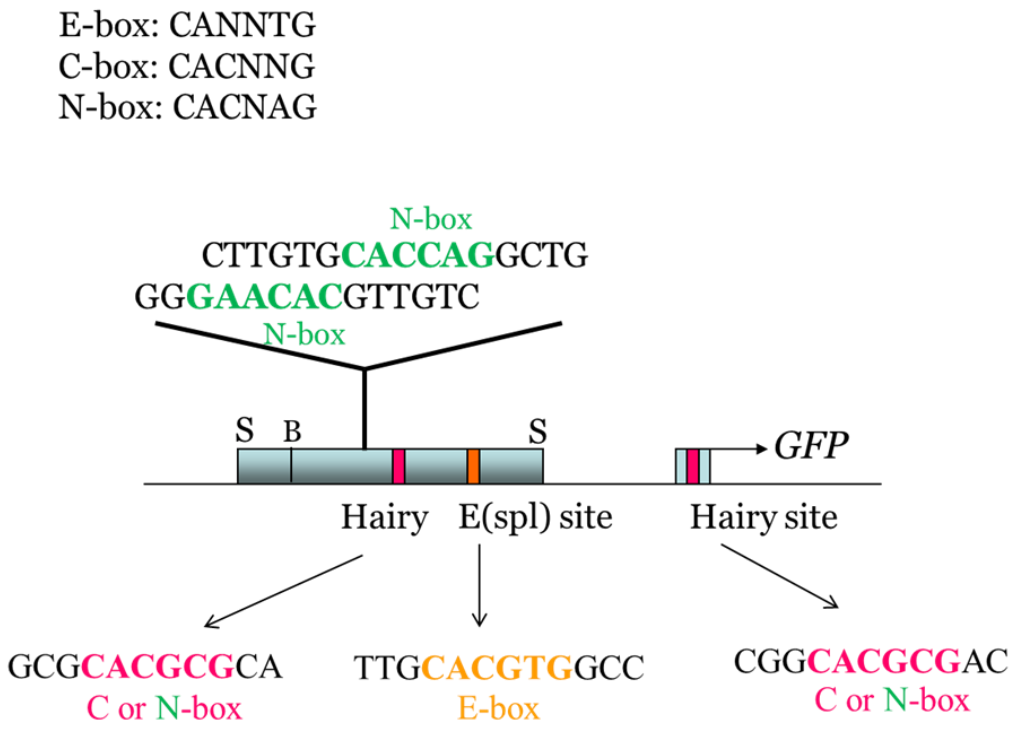


Figure 24. Potential Hairy and Notch signaling response sites in the *ac* mC-CRE and *ac* promoter.

bHLH repressors have been shown to bind a C-box (CACNNG) or an N-box (CACNAG). Hairy is a bHLH repressor that has been shown to bind preferentially to a specific C-box (CACGCG) (Ohsako et al., 1994; van Doren et al., 1994). The E(spl) protein, which have similar DNA binding specificity, bind a palindromic sequence, TGG**CACGTG**C/TC/TA, which has a C/E-box (E-box: CANNTG) core, CACGTG (Jennings et al., 1999). A Hairy binding sequence in the ac promoter is known to repress *ac* expression in the wing disc. In addition, we have identified putative Hairy and E(spl) sites and two N-boxes in the *ac*-mC-CRE.

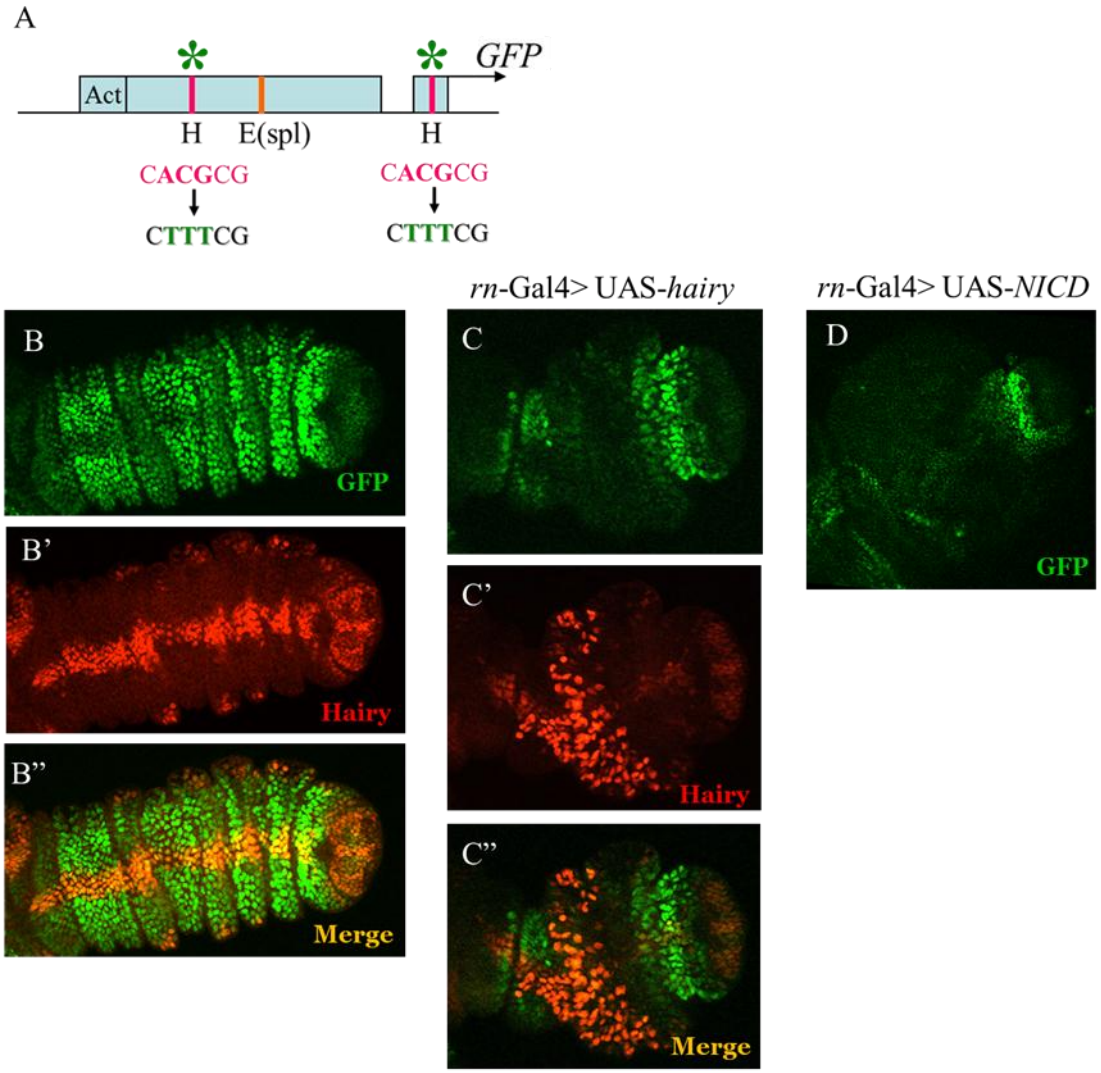
Figure 25. The *ac* promoter and *ac*-mC-CRE putative Hairy binding sites are required for full response to Hairy-mediated repression.

(A) Schematic map of the *ac*-GFP-FL-H1*H2* transgene. This reporter construct was generated by introducing mutations in the *ac* promoter and *ac*-mC-CRE Hairy sites (H1 and H2, respectively, see text) of the *ac*-GFP-FL transgene (Figure 11). The asterisks designates that the H1 and H2 sites have been mutated.

(B-B") As compared to the *ac*-GFP-FL transgene, GFP expression (green in B, B'') directed by the *ac*-GFP-FL-H1*H2* transgene is elevated in the *hairy*-On interstripes and also expands into the the *hairy*-Off interstripes (anti-Hairy, red in B', B'') in prepupal legs.

(C-C") In prepupal legs, ectopic and overexpression of Hairy causes reduces GFP expression (green in C, C'') directed by the *ac*-GFP-FL-H1*H2* transgene in Hairy-expressing cells (anti-Hairy, red in C', C''). This suggests that, despite the mutations in the H1 and H2 sites, this transgene is Hairy-repsonsive.

(D) Ectopic activation of N signaling also reduces GFP expression (green in C, C'') directed by the *ac*-GFP-FL-H1*H2*.


A
GFP
Act
H
E(spl)
H
CACGCG
CTTTCG
CACGCG
CTTTCG
rn-Gal4> UAS-hairy
rn-Gal4> UAS-NICD
B
GFP
B'
Hairy
B"
Merge
C
C'
Hairy
C"
Merge
D
GFP

sites within the repression element. We favor the latter interpretation because in the analysis of the Hairy binding site in the promoter, the same changes were made to the sequence and we observed complete loss of response to Hairy. These observations suggest that the wild type levels of endogenous Hairy are not sufficient to fully repress *ac* expression, when the function of the H1 and H2 sites is compromised, but the super-physiological levels produced via the UAS/Gal4 system can compensate for the loss of the two sites.

Adult legs from *Dfsc10-1/Y* flies carrying the *ac*-Res-FL-H1*H2* transgene exhibit mildly disorganized L-rows and ectopic mC bristles between the rows, consistent with our conclusion that Hairy response has been partially compromised (Figure 26D). On the other hand, no phenotype was observed in wild type wings carrying the *ac*-Res-FL-H1*H2* (Figure 26E-E'). Combined, our observations indicate that the H1 and H2 sites together are required for full response to Hairy-mediated repression. All the results of transgenic fly lines are summarized in Table 3 for the rescue constructs and in Table 4 for the GFP reporter constructs. As will be discussed in subsequent sections, we have identified an additional sequence in the repression element that is responsive to Hairy.

4.4. Dl/N-mediated repression of *ac* expression in the leg

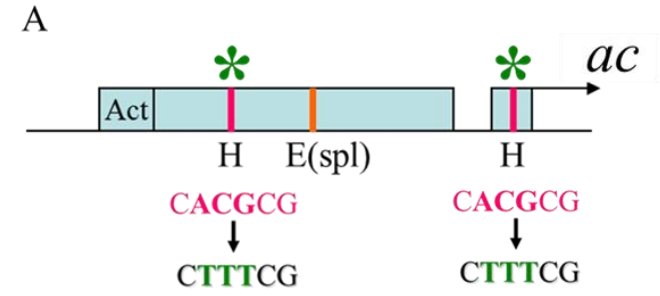
Previous genetic and molecular data have shown that Dl/N signaling is responsible for repression of *ac* in the *hairy*-Off interstripes (Joshi et al., 2006). In the prepupal leg, *Dl* is expressed in eight longitudinal stripes overlapping Ac expression stripes in the mC proneural fields and signals to adjacent cells to activate N signaling in the *hairy*-Off interstripes, in which N turns on one or more gene(s) of the *Enhancer of split* complex [*E(spl)*-C]

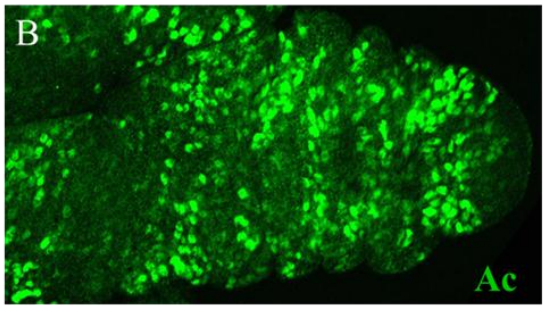
Figure 26. The *ac*-Res-FL-H1*H2* rescue transgene directs expanded *ac* expression into *hairy*-Off interstripes in prepupal legs and ectopic *ac* expression wing discs.

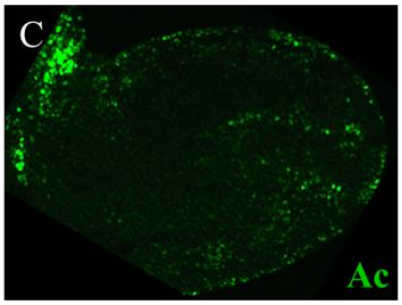
(A) Schematic map of the *ac*-Res-FL-H1*H2* transgene. This reporter construct was generated by introducing mutations in the *ac* promoter and *ac*-mC-CRE Hairy sites (H1 and H2, respectively, see text) of the *ac*-REs-FL transgene (Figure 11). The asterisks designates that the H1 and H2 sites have been mutated.

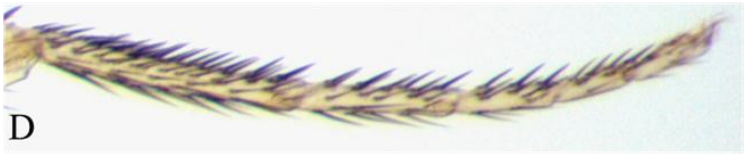
(B and D) A $y^{3PL}sc^{8R}$/Y prepupal leg (B) carrying the *ac*-Res-FL-H1*H2* rescue transgene exhibits expanded, almost uniform, Ac expression. A $Dfsc^{10\text{-}1}$/Y adult prepupal leg (C) carrying the *ac*-Res-FL-H1*H2* exhibits rescue of many mC and als0 ectopic mCs.

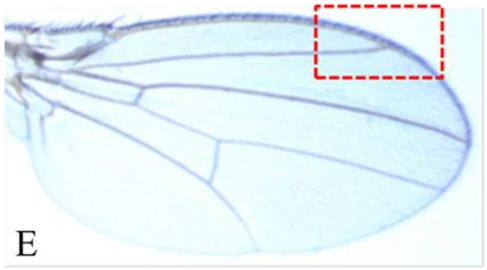
(C and E) A $y^{3PL}sc^{8R}$/Y prepupal wing (C) carrying the *ac*-Res-FL-H1*H2* rescue transgene exhibits ectopic Ac expression in a few wing blade cells. However, no phenotype is observed in $Dfsc^{10\text{-}1}$/Y adult legs (D) carrying the *ac*-Res-FL-H1*H2* transgene.

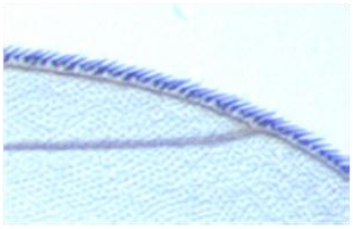

A
ac
Act
H
E(spl)
H
CACGCG
CTTTCG
CACGCG
CTTTCG


B
Ac


C
Ac


D


E



(Bailey and Posakony, 1995; Lecourtois and Schweisguth, 1995) (Joshi et al., 2006; Parks et al., 1997). Like *hairy*, the *E(spl)-C* genes encode bHLH repressors, which are known to repress proneural gene expression (Giagtzoglou et al., 2003). In this section, we discuss N-mediated repression of *ac* via the *ac* promoter and the *ac*-mC-CRE.

4.4.1. The *achaete* promoter Hairy-binding site is responsive to Dl/N signaling

Our initial analysis of Dl/N mediated regulation of *ac* expression in the mC pronerual fields suggests that Dl/N signaling goes primarily through the *ac*-mC-CRE repression element. This is suggested by the observation that *ac*-GFP-Act directs GFP expression in the *hairy*-Off interstripes. However, we find that overexpression of the constitutively active form of N (UAS-*NICD*), under the control of *rn*-Gal4, compromises expression from the *ac* mC-CRE transgene (Figure 16C-C"). In interpreting this finding, it is important to consider the binding specificity of the E(spl) proteins. A previous study has shown that the different E(spl) proteins bind the same palindromic sequence, TGG**CACGTG**T/CT/CA, which has an E-box core (Jennings et al, 1999). This study demonstrated that the E(spl) proteins preferentially bind an E-box core over a C-box, the preferred binding sequence for the Hairy repressor. The E-box (CANNTG) is a core binding sequence for bHLH proneural activators, such as Ac and Sc, as well as bHLH repressors, such as the E(spl) proteins. It has been suggested that the E(spl) proteins and proneural activators preferentially bind different E-boxes, although there are E-boxes that could be bound by both. The *ac* promoter has, in addition to the Hairy binding C-box, three E-boxes that mediate Ac autoregulation.

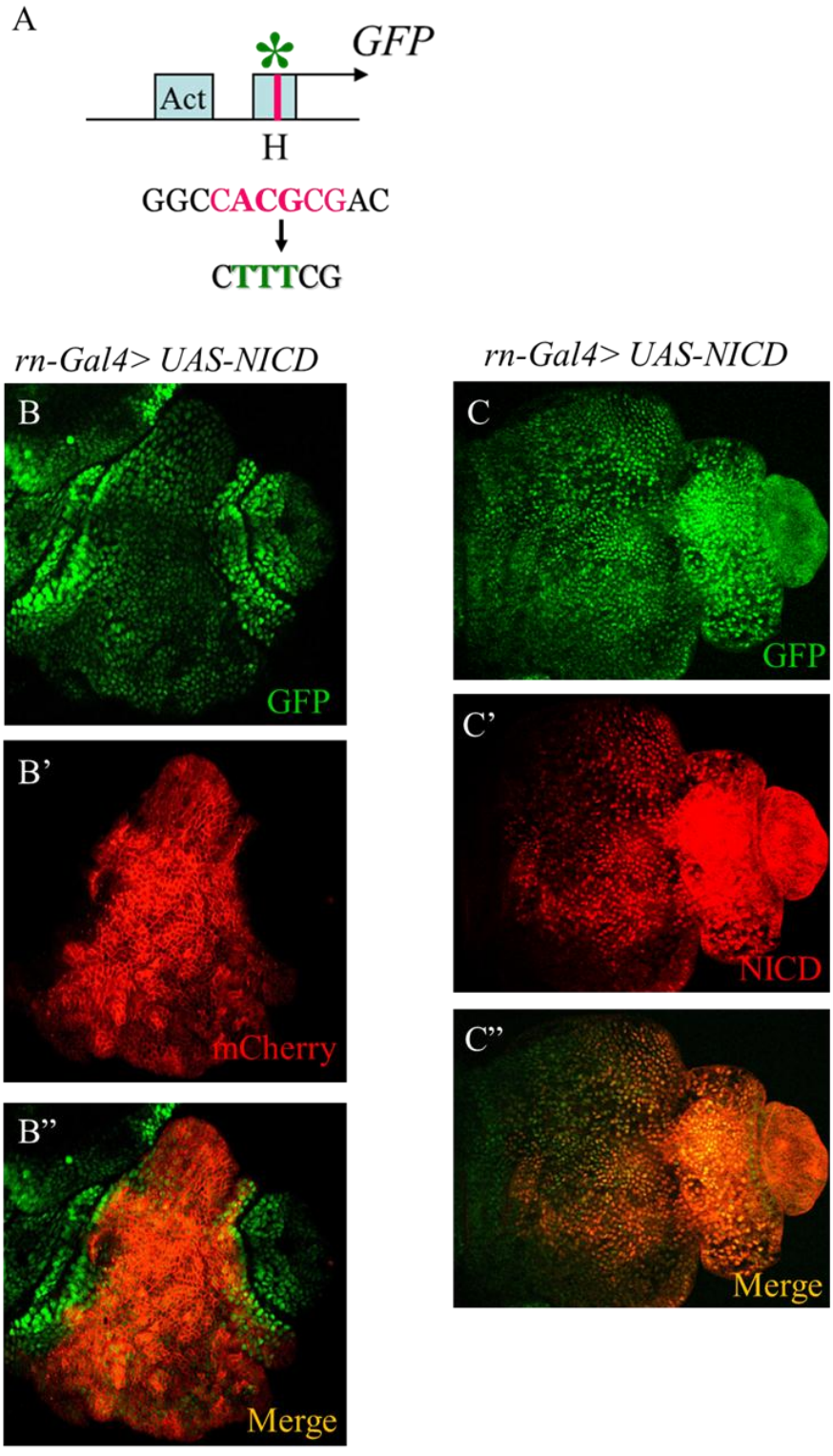
Based on these prior studies, one interpretation of our findings is that activation of N signaling at high levels, results in expression of one more E(spl) proteins, which inhibit

Figure 27. The *achaete* promoter Hairy-binding site is responsive to Delta/Notch signaling.

(A) Schematic map of the *ac*-GFP-Act-H1* transgene. This reporter construct was generated by introducing a mutation in the Hairy binding sequence (see text) in the *ac* promoter (H1) of the *ac*-GFP-Act transgene (Figure 11). The asterisk designates that the H1 site has been mutated.

(B-B") A *rn-Gal 4* driver was used to direct UAS-*NICD(III)* and UAS-*mCherry* (B', B'') in mid-prepupal legs carrying the *ac*-GFP-Act transgene, GFP expression was lost (green in B, B"). This suggests that *ac*-GFP-Act transgene is responsive to Notch signaling. A potential explanation for the discrepant results is provided in the text.

(C-C") On the other hand, expression of UAS-*NICD(III)* under control of *rn*-Gal4, in a prepupal leg carrying the *ac*-GFP-Act-H1* transgene does not result in compromised GFP expression (green in C, C") in NICD expressing cells (anti-NICD, red in C' and C"). This result indicates that Notch signals through the Hairy binding site in the *ac* promoter.


A
GFP
Act
H
GGCCACGCGAC
CTTTCG
rn-Gal4> UAS-NICD
B
GFP
B'
mCherry
B''
Merge
rn-Gal4> UAS-NICD
C
GFP
C'
NICD
C''
Merge

expression of the *ac*-*GFP*-Act transgene by binding one of three E-boxes in the *ac* promoter. Alternatively, it is plausible that Dl/N signaling goes through the Hairy site, even though the C-box core is not the optimal binding sequence for the E(spl) proteins. To distinguish between these alternatives, we tested the response of *ac*-*GFP*-Act-H1* to ectopic Dl/N signaling. We find that mutation of the H1 sites results in loss of the *ac*-*GFP*-Act response to Dl/N signaling (Figure 27C-C"), indicating that the E(spl) proteins likely act through the H1 site, instead of the E-boxes in the *ac* promoter.

4.4.2. A putative E(spl) site in the *ac* mC-CRE is not essential for repression mediated by Dl/N signaling

Our analysis of the response of the *ac* promoter to Dl/N signaling indicates that, although the Hairy-binding sequence can respond to high-level Dl/N signaling, it is likely that additional sequences in the *ac*-mC-CRE repression element are required. As shown in Figure 24, there is a putative E(spl) binding site, TTG**CACGTG**CC within the *ac* mC-CRE. This site matches the E(spl) consesnsus binding sequence in 9/12 nucleotides and the core sequence, CACGTG, corresponds to an E or C-box, although it differs from the C-box in the Hairy site core, CACGCG. Although the putative E(spl) site is not conserved, it exactly matches the E(spl) consensus core sequence and matches 75% of the consensus sequence, overall. We, therefore, opted to assay the potential function of this sequence in mediating response to Dl/N signaling.

Our initial mutation of the putative E(spl) binding site generated another E-box sequence (CACGTG to CATTTG). This mutation had no effect on *ac*-GFP-FL expression or

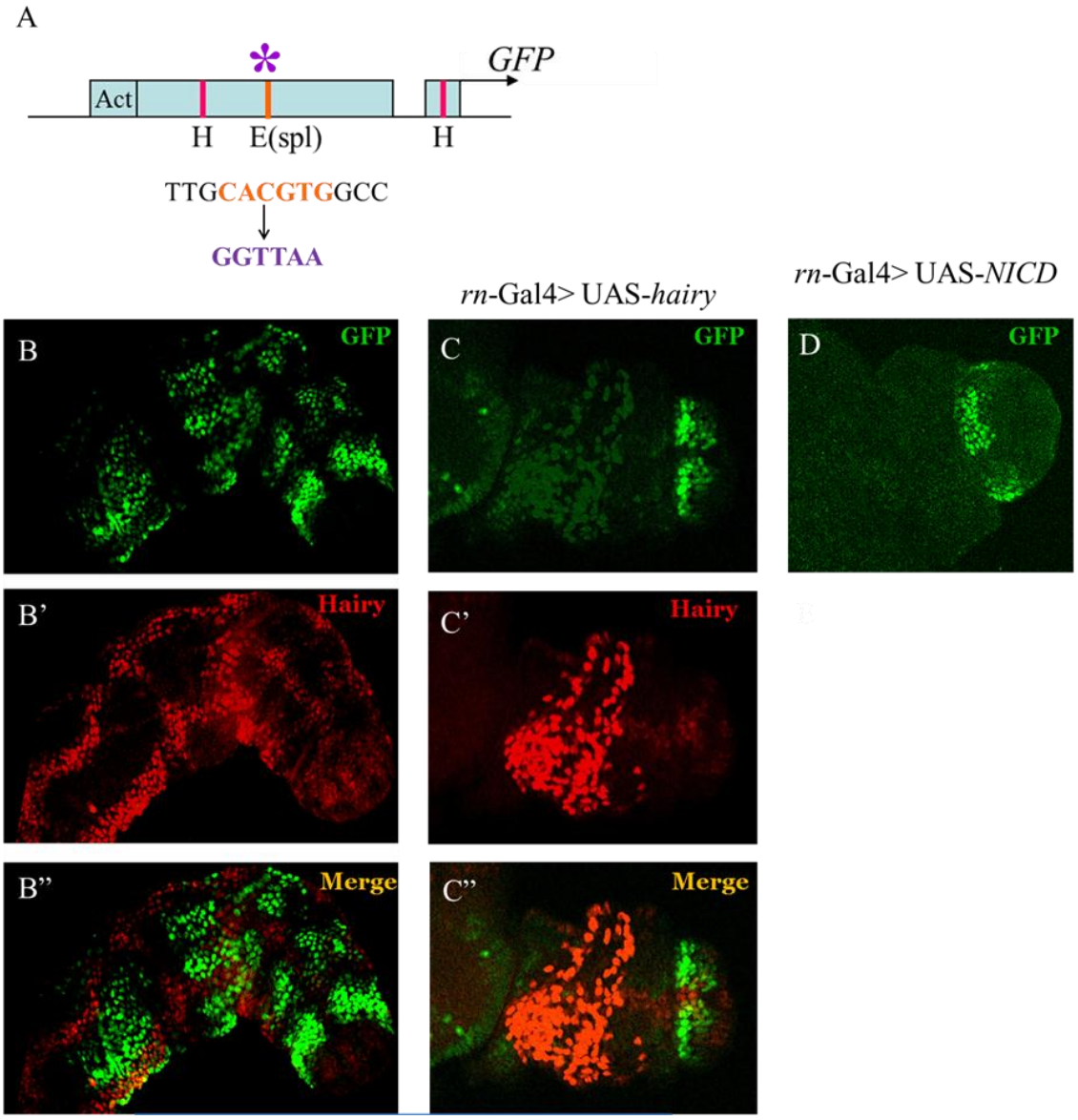
Figure 28. A putative E(spl) site in the *ac* mC-CRE is not essential for repression mediated by Delta/Notch signaling.

(A) Schematic map of the *ac*-GFP-FL-E* transgene. This reporter construct was generated by introducing a mutation in the putative E(spl) binding sequence (see text) in the *ac*-mC-CRE of the *ac*-GFP-FL transgene (Figure 11). The asterisk designates that the E(spl) site has been mutated.

(B-B") In prepupal legs, the *ac*-GFP-FL-E* transgene directs GFP (green in B, B") expression in narrow longitudinal stripes as observed in wild type prepupal legs. The *hairy-On* interstripes are marked by anti-Hairy (red in B'-B").

(C-C") Overexpression of *hairy* (UAS-hairy) under control of *rn*-Gal4 results in loss of GFP expression (green in C, C'') in Hairy expressing cells (red in C',C'') suggesting the *ac*-GFP-FL-E* reporter construct is Hairy responsive.

(D) Overexpression of *NICD* under control of *rn*-Gal4 results in loss of GFP expression in the *rn* expression domain, suggesting the *ac*-GFP-FL-E* reporter construct is Notch responsive.


A
GFP
Act
H
E(spl)
H
TTGCACGTGGCC
GGTTAA
rn-Gal4> UAS-hairy
rn-Gal4> UAS-NICD
B
GFP
C
GFP
D
GFP
B'
Hairy
C'
Hairy
B"
Merge
C"
Merge

response to N signaling (not shown). Therefore, a new mutation (CA**CGT**G to GGTTAA, Figure 28A and 29A), which disrupted the E-box, was introduced and reporter (*ac*-*GFP*-FL-E*) and rescue transgenes (*ac*-Res-FL-E*) were generated. Mutation of the putative E(spl) binding site appears to have a mild or no effect on the activity of the *ac*-mC-CRE, as GFP expression from *ac*-*GFP*-FL-E* transgene appears similar to *ac*-*GFP*-FL (compare Figures 28B and 16A). Additionally, the *ac*-*GFP*-FL-E* is responsive to N-signaling (Figure 28D).

The *ac*-Res-FL-E* transgene exhibits a wild type pattern of *ac* expression and promotes formation of a wild type mC pattern in legs Df*sc*$^{10\text{-}1}$ males (Figure 29C). Also, no ectopic mC bristles are observed in wings from wild type animals carrying the transgene. Together, these observations suggest either that the putative E(spl) binding site does not mediate response to Dl/N signaling or it is not essential for N signaling, due to the existence of redundant sites. All the results of transgenic fly lines are summarized in Table 3 for the rescue constructs and in Table 4 for the GFP reporter constructs. In the following section, we discuss the identification of additional putative Dl/N response sequences.

4.5. Identification of additional Hairy and E(spl) response sequences

Thus far, we have tested the function of two putative Hairy-binding sites and a putative E(spl) site in mediating repression of *ac* expression. Although our analysis suggests that the Hairy-binding site in the *ac* promoter (H1) and a putative Hairy-binding sequence in the *ac*-mC-CRE (H2), are responsive to Hairy-mediated repression, (Section 4.3.3 and 4.3.4), there is some evidence that there are additional Hairy response sites in the *ac*-mC-CRE repression element. On the other hand, our studies do not provide convincing evidence that

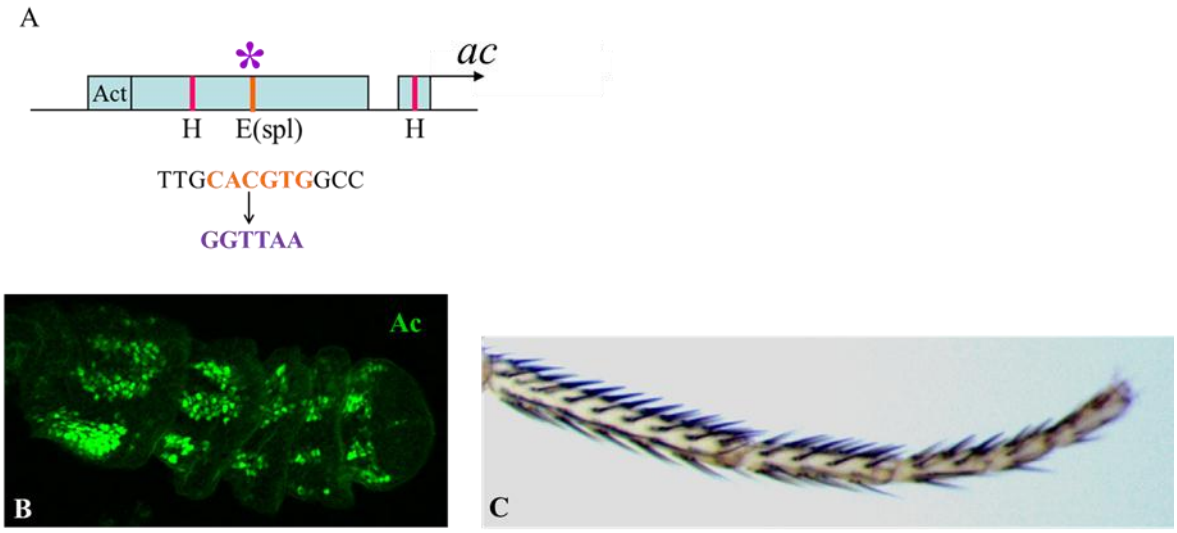


Figure 29. A putative E(spl) site in the *ac* mC-CRE is not essential for repression mediated by Delta/Notch signaling.

(A) Schematic map of the *ac*-Res-FL-E* transgene. This reporter construct was generated by introducing a mutation in the putative E(spl) binding sequence (see text) in the *ac*-mC-CRE of the *ac*-Res-FL transgene (Figure 11). The asterisk designates that the E(spl) site has been mutated.

(B) The *ac*-Res-FL-E* transgene directs Ac expression (anti-Ac, green) in four narrow longitudinal stripes in $y^{3PL}sc^{8R}$/Y prepupal legs.

(C) A $Dfsc^{10\text{-}1}$/Y adult leg carrying the *ac*-Res-FL-E* transgene does not show any ectopic mCs or disrupted mC pattern.

the E(spl) site mediates response to Dl/N signaling (Section 4.4.2). Therefore, we sought to identify additional sites that mediate response to Hairy and Dl/N mediated repression. We reasoned that since there is substantial similarity between the Hairy and E(spl) consensus sequences, and since Dl/N can signal through the H1 site in the *ac* promoter, it is plausible that Hairy and E(spl) act through common sequences to repress *ac* expression. This possibility is explored in the following section.

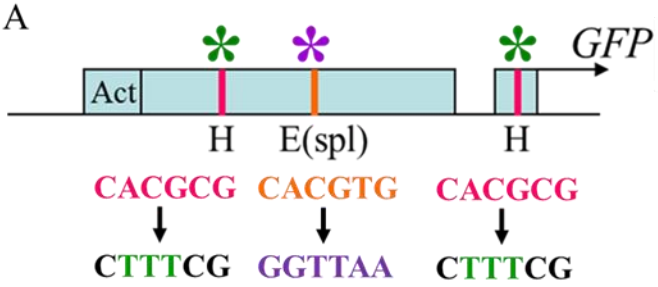
4.5.1. Hairy and Dl/N act through common sites to repress *ac* expression

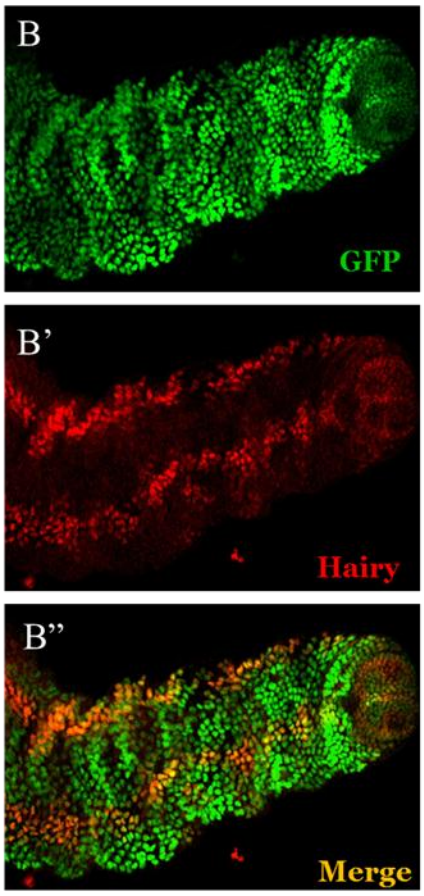
To determine whether Hairy and E(spl) act through common sequences to repress proneural *ac* expression, we introduced mutations in the H1 site in the *ac* promoter, and the putative H2 site and the E(spl) site in the *ac*-mC-CRE. Rescue, *ac*-Res-FL-H1*H2*E*, and reporter, *ac*-GFP-FL-H1*H2*E*, transgenes carrying the triple mutations were generated (Figure 30A and 32A). We were surprised to observe that the triply mutant transgenes appear to be compromised in their response to Hairy (Figure 30B-B" and 32B). Note in Figure 30B-B", that GFP expression overlaps Hairy expression and compare to expression from the *ac*-GFP-FL-H1*H2* transgene in Figure 25B-B'', in which *ac* expression appears to be partially repressed in the Hairy interstripes. This suggests that the E(spl) site is responsive to Hairy. Furthermore, ectopic mCs are observed in $Dfsc^{10\text{-}1}$/Y flies carrying the *ac*-Res-FL-H1*H2*E* transgene (Figure 32C-F'). Even more compelling is that *ac* is ectopically expressed in prepupal wings from wild type animals carrying the *ac*-Res-FL-H1*H2*E* transgene, and ectopic mCs are formed in the wing blades of adults (Figure 32E-E'). Wings from animals carrying *ac*-Res-FL-H1*H2* transgene, on the other hand, do not exhibit

Figure 30. A combination of Hairy and E(spl) sites mediates repression by Hairy and Notch signaling.

(A) Schematic map of the *ac*-GFP-FL-H1*H2*E* transgene. This reporter construct was generated by introducing mutations in the E(spl) and Hairy (E* and H2*, respectively) sites of the *ac*-mC-CRE in the *ac*-GFP-FL transgene and the Hairy site in the *ac* promoter (H1*).

(B-B") The *ac*-GFP-FL-H1*H2*E* transgene (shown in A) directs uniform GFP expression along the prepupal leg circumference, overlapping *hairy*-On (anti-Hairy, in red) and *hairy*-Off interstripes, suggesting that response to both Hairy and Notch signaling has been compromised.


A
Act
GFP
H
E(spl)
H
CACGCG
CACGTG
CACGCG
CTTTCG
GGTTAA
CTTTCG


B
GFP
B'
Hairy
B''
Merge

ectopic mC bristles (Figure 26E-E'). In addition, in the notum, Hairy, but not N, is known to repress *ac* expression in the scutellar primordia, and *hairy* mutants exhibit ectopic mC bristles in the scutellum. We observe ectopic mC bristles on the scutellum of animals carrying the *ac*-Res-FL-H1*H2*E* transgene (Figure 26F-F'). Taken together, these observations suggest that Hairy functions through the putative E(spl) binding site, in addition to the H1 and H2 sites. However, *ac*-Res-FL-H1*H2*E* is still responsive to ectopically expressed Hairy (Figure 31), suggesting the existence of additional Hairy response sequences in the *ac*-mC-CRE.

The triply mutant transgenes also appear to be compromised in their response to Dl/N signaling, as *ac* and GFP expression from the *ac*-Res-FL-H1*H2*E* and *ac*-GFP-FL-H1*H2*E* insertions, respectively, expands into the *hairy*-Off interstripes (Figure 30B-B" and 32B). Given that the E(spl) site is dispensable for response to N-signaling, this would imply that N signals through one or both of the Hairy sites. However, as observed with Hairy, ectopic activation of N signaling abrogates *ac*-GFPs-FL-H1*H2*E* expression, suggesting that there are additional N-responsive sites in the *ac* mC-CRE (Figure 31C).

Our findings suggest that the Hairy and Dl/N response is mediated by common binding sites. The H1 site in the *ac* promoter is responsive to Hairy and to high-level Dl/N signaling. In addition, the analysis of the triply mutant transgenes suggests that the *ac*-mC-CRE H2 site is also responsive to both Hairy and Dl/N. On the other hand, the E(spl) sequence appears to be Hairy responsive, but it is less clear if it is also Dl/N responsive. In the following sections, we further explore the functions of the H2 and E(spl) binding sites in Hairy and Dl/N mediated repression.

Figure 31. The *ac*-GFP-FL-H1*H2*E* transgene is responsive to Hairy and Notch signaling.

(A) Schematic map of the *ac*-GFP-FL-H1*H2*E* transgene. This reporter construct was generated by introducing mutations in the E(spl) and Hairy (E* and H2*, respectively) sites of the *ac*-mC-CRE in the *ac*-GFP-FL transgene and the Hairy site in the *ac* promoter (H1*).

(B-B") Ectopic and overexpression of *hairy* in prepupal legs carrying the *ac*-GFP-FL-H1*H2*E* transgene results in loss of GFP expression (green in B, B'') in Hairy expressing cells (red in B', B''), suggesting the *ac*-GFP-FL-H1*H2*E* transgene is Hairy responsive.

(C) Overexpression of *NICD* under control of *rn*-Gal4 in prepupal legs carrying the *ac*-GFP-FL-H1*H2*E* transgene, results in loss of GFP expression in the *rn* expression domain, suggesting that the *ac*-GFP-FL-H1*H2*E* transgene is Notch responsive.

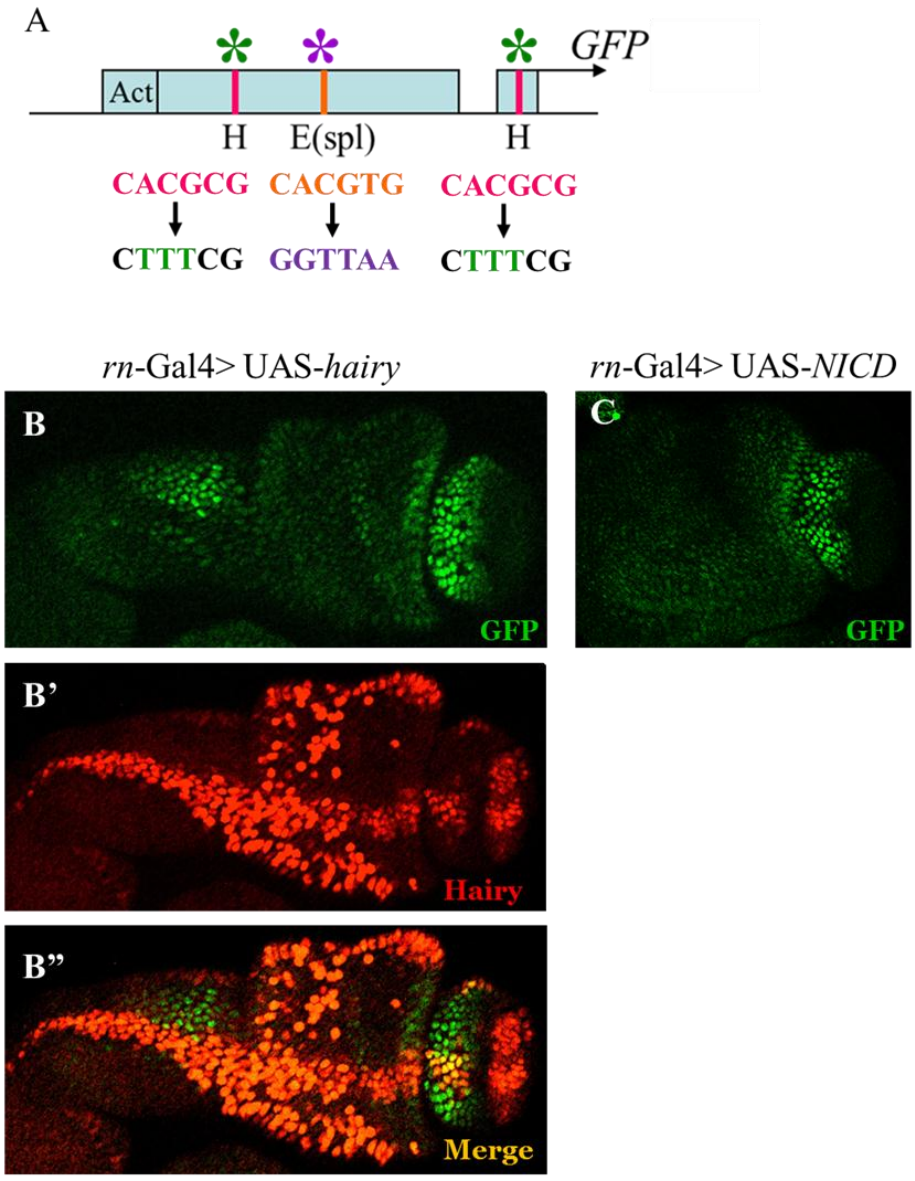

A
GFP
Act
H
E(spl)
H
CACGCG
CACGTG
CACGCG
CTTTCG
GGTTAA
CTTTCG
rn-Gal4> UAS-hairy
rn-Gal4> UAS-NICD
B
GFP
C
GFP
B'
Hairy
B"
Merge

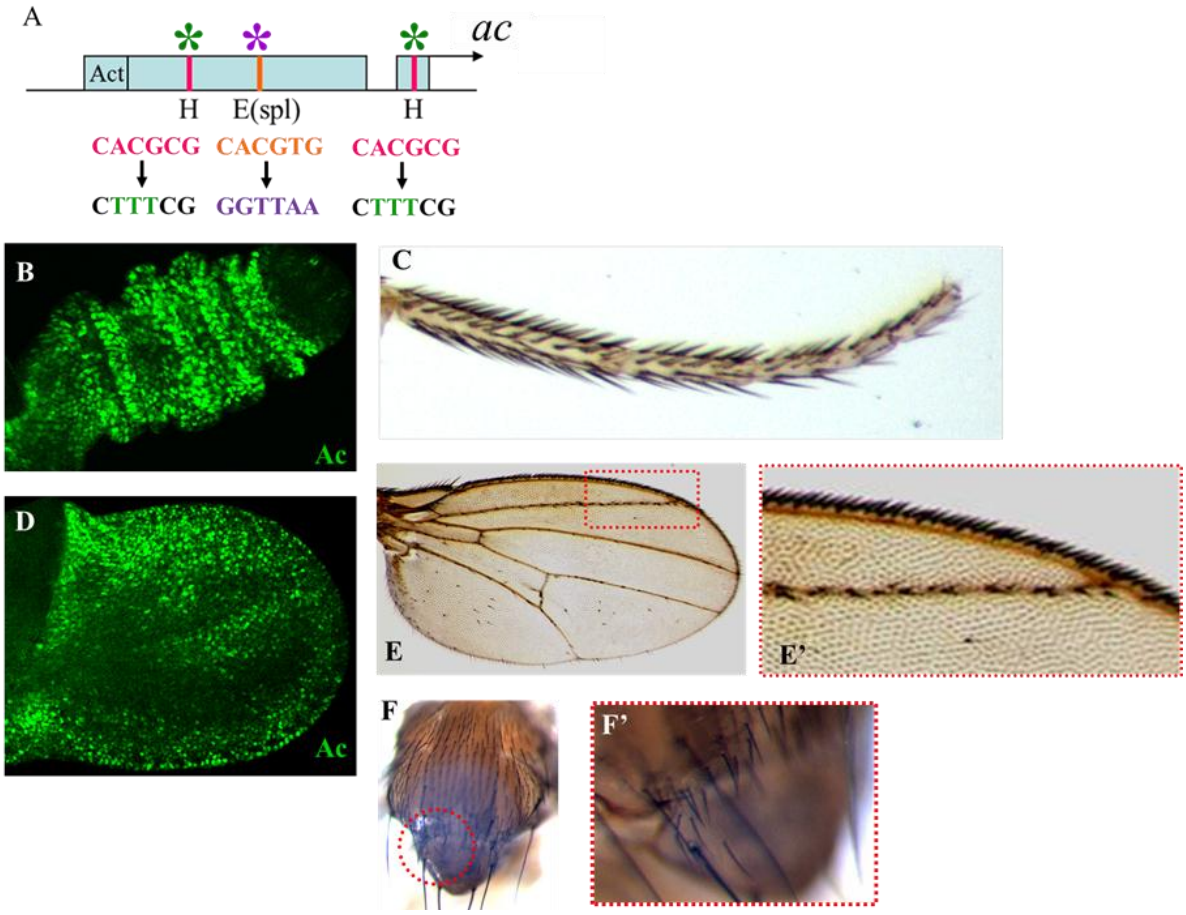
Figure 32. A combination of Hairy and E(spl) sites mediates repression by Hairy and Notch signaling.

(A) Schematic map of the *ac*-Res-FL-H1*H2*E* transgene. This reporter construct was generated by introducing mutations in the E(spl) and Hairy (E* and H2*, respectively) sites of the *ac*-mC-CRE in the *ac*-Res-FL transgene and the Hairy site in the *ac* promoter (H1*)..

(B and D) Prepupal legs (B) and wings (D) from $y^{3PL}sc^{8R}$/Y flies carrying the *ac*-Res-FL-H1*H2*E* transgene shown in (A). The rescue construct directs uniform Ac expression along the prepupal leg circumference and in the wing (anti-Ac, in green), suggesting compromised response to Hairy and Notch signaling.

(C) $Dfsc^{10\text{-}1}$/Y adult leg carrying the *ac*-Res-FL-H1*H2*E* transgene shown in (A). Rescue of most of the mCs and ectopic mCs are observed in the leg.

(D-E') Adult wild type wing (E) and notum (F) carrying the *ac*-Res-FL-H1*H2*E* transgene exhibit ectopic mC bristles in the wing blade and scutellum, respectively, phenocoyping the *hair*y mutant phenotype in these tissues. A portion of wing and notum is magnified in E' and F', respectively. These observations provide strong support for the suggesting that E(spl) sequence mediates response to Hairy (see text).


A
ac
Act
H
E(spl)
H
CACGCG
CACGTG
CACGCG
CTTTCG
GGTTAA
CTTTCG
B
Ac
C
D
Ac
E
E'
F
F'

4.5.2. The putative Hairy site in the *ac* mC-CRE is responsive to Hairy and Dl/N

Mutations in H1 and H2 sites do not completely abolish Hairy responsiveness, suggesting that Hairy can repress *ac* expression through other sequences. Our analysis, described above, suggests that one additional site through which Hairy acts is the putative E(spl) site, TTG**CACGTG**[C/T][C/T], which has a core sequence (shown in bold) that is both an E-box and C-box (Jennings et al., 1999). It has been shown that Hairy binds a site containing the C-box sequence, CACGCG, with high affinity (Ohsako et al., 1994; Van Doren et al., 1994). This sequence differs by one nucleotide from the C-box in the E(spl) site.

To directly test the responsiveness of the conserved H2 site to Hairy and Dl/N, a 40 bp oligo consisting of the H2 site and neighboring sequences (see Materials and Methods) was cloned 3' to the *ac*-GFP-Act-H1* reporter gene (Figure 33A), and transgenic flies carrying this construct, *ac*-*GFP*-Act-H1*+H2, were made. The *ac*-GFP-Act-H1*+H2 transgene appears to slightly respond to endogenous Hairy (Figure 33B-B"). However, over- and ectopic expression of Hairy results in almost complete elimination of GFP expression (Figure 33C-C"). As previously described, the *ac*-GFP-Act-H1* transgene, which differs from *ac*-GFP-Act-H1*+H2 only by the presence of the H2 oligo, fails to respond to Hairy. This finding strongly indicates that the H2 site is Hairy-responsive. Furthermore, the *ac*-GFP-Act-H1*+H2, but not the *ac*-GFP-Act-H1*, transgene shows responsiveness to N signaling (Figure 33D), consistent with our previous observations suggesting that the H1 and H2 sites are responsive to N signaling, as well Hairy.

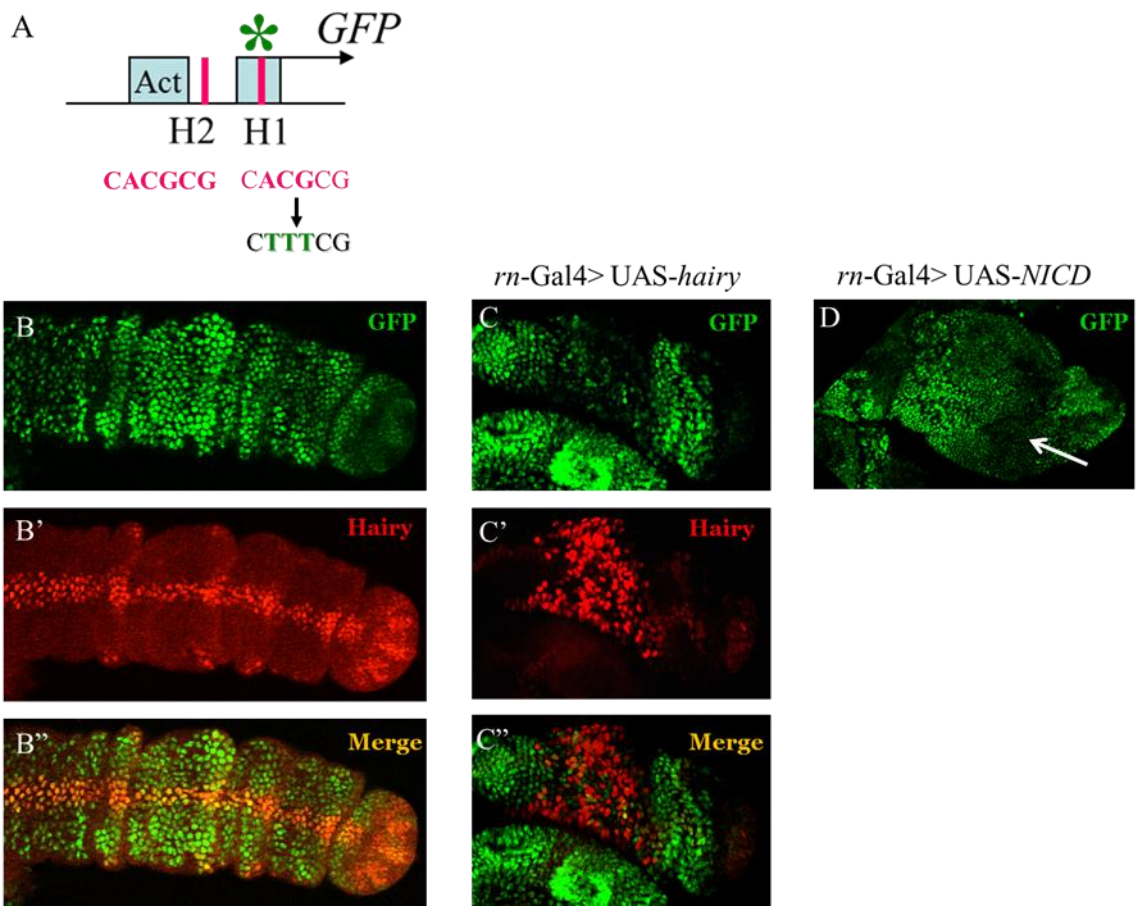
Figure 33. A putative Hairy site in the *ac*-mC-CRE mediates repression by Hairy, as well as Notch signaling.

(A) Schematic map of the *ac*-GFP-Act-H1*+H2 transgene. This reporter construct was generated by cloning a 40 bp oligo containing the H2 site and adjacent sequences (see Materials and Methods) just 3' to the *ac*-GFP-Act-H1* transgene.

(B-B") GFP expression (green in B, B'') in prepupal leg carrying the *ac*-GFP-Act-H1*+H2 transgene appears to be broadly activated along the leg circumference, partially overlapping the Hairy expressing interstripes (anti-Hairy, red in B', B'').

(C-C") Ectopic and overexpression of Hairy (anti-Hairy, red in C', C'') results in reduced GFP expression (green in C, C'') in Hairy-expressing cells, suggesting the *ac*-GFP-Act-H1*+H2 transgene is responsive to Hairy due to the addition of Hairy site.

(D) Overexpression of *NICD* under control of *rn*-Gal4 results in reduced GFP expression in the *rn* expression domain (indicated by a white arrow), suggesting that the *ac*-GFP-Act-H1*+H2 transgene is responsive to Notch signaling and,therefore, Notch can mediate repression through the Hairy site in the *ac*-mC-CRE.


A
GFP
Act
H2
H1
CACGCG
CACGCG
CTTTCG
rn-Gal4> UAS-hairy
rn-Gal4> UAS-NICD
B
GFP
C
GFP
D
GFP
B'
Hairy
C'
Hairy
B"
Merge
C"
Merge

4.5.3. The putative E(spl) site in the ac mC-CRE is responsive to Hairy but not to Dl/N

In order to test whether Hairy and/or N-mediated repression go through the putative E(spl) site, a 40 bp oligo consisting of the E(spl) binding site and neighboring sequences (see Materials and Methods) was cloned 3' to the *ac*-GFP-Act-H1* reporter gene, as described above for the H2 oligo, to generate an *ac*-GFP-Act-H1*+E reporter gene (Figure 34A). The *ac*-GFP-Act-H1*+E reporter gene drives uniform GFP expression along the leg circumference, suggesting it is not responsive to endogenous levels of Hairy or Dl/N signaling (Figure 34B). However, it does respond to ectopically expressed Hairy (Figure 34D-D"), but, surprisingly, not to ectopically activated N signaling (Figure 34C). These observations suggest that repression by Hairy can go through this sequence. On the other hand, despite its similarity to the E(spl) consensus sequence, this site apparently does not respond to Dl/N signaling.

4.6. Discussion

4.6.1. Global activation of *achaete* expression

We have proposed a model for regulation of *ac* expression in the mC proneural fields, which suggests that *ac* expression is activated broadly throughout prepupal legs by a uniformly expressed activator(s) and that expression is refined to the mC proneural fields via repressive inputs from Hairy and Dl/N signaling. This proposed mechanism for *ac/sc* regulation in the mC proneural fields is distinct from that used to establish expression of *ac* expression in the MC proneural clusters. *ac* expression in the MC proneural clusters is controlled by locally expressed activators and repressors that act through several modular

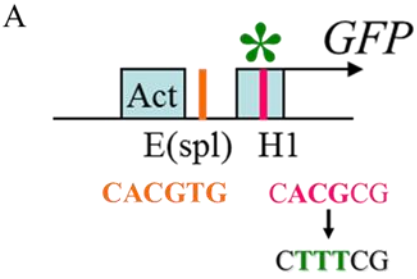
Figure 34. A potential E(spl) binding site mediates repression by Hairy but not Notch signaling.

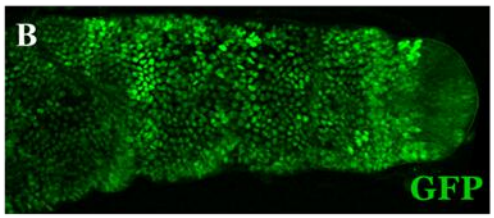
(A) Schematic map of the *ac*-GFP-Act-H1*+E transgene. This reporter construct was generated by cloning a 40 bp oligo containing the E(spl) site and adjacent sequences (see Materials and Methods) just 3' to the *ac*-GFP-Act-H1* transgene.

(B) GFP expression (green in B, B'') in prepupal legs carrying the *ac*-GFP-Act-H1*+E transgene appears to be broadly activated along the leg circumference.

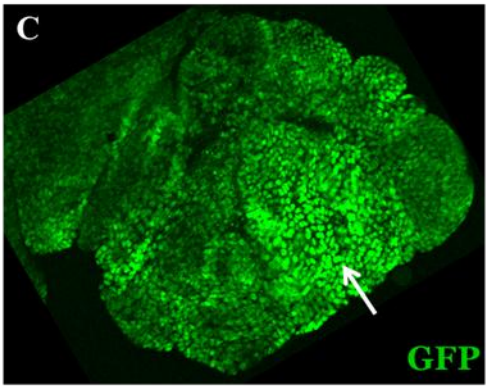
(C) Overexpression of *NICD* (UAS-*NICD*) under control of *rn*-Gal4 does not result in loss of GFP in the *rn* expression domain (indicated by a white arrow), suggesting that the tested E(spl) site does not mediate repression by Notch signaling.

(D-D") Ectopic and overexpression of Hairy results in reduced GFP expression (green in D, D'') in Hairy expressing cells (anti-Hairy, red in D', D''), suggesting that the potential E(spl) site can mediate repression by Hairy.





rn-Gal4> UAS-*NICD*



rn-Gal4> UAS-*hairy*

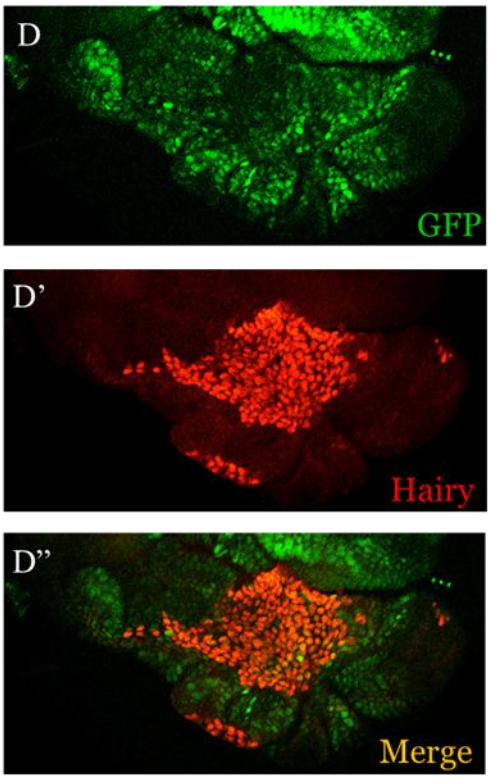


Table 3. Summary of phenotypes and expression patterns of the rescue transgenic fly lines

Transgenic rescue line	mC Rescue Leg	mC Rescue Notum	Ectopic bristles in the wing	Ac expression
ac-Res1	+++	+++	-	+++
ac-Res3	++	++	-	++
ac-Res2	+	+	-	+
ac promoter - *ac*	-	-	-	ND
ac-Res4	+++	+++	-	+++
ac-Res5 mC CRE Act	++	+++	-	+
ac-Res-Act-H1*	+++	+++	+++	++++
ac-Res-FL-H1*	+++	+++	-	+++
ac-Res-FL-H1*H2*	+++	+++	-	+++
ac-Res-FL-E*	+++	+++	-	+++
ac-Res-FL-H1*H2*E*		++++	++	++++

++++ Rescue with ectopic bristles/ Uniform Ac expression

+++ Rescue as exhibited in wild type/ Wild type Ac expression

++ Partial rescue/ Partial Ac expression

+ Rescue of a few bristles/ Some Ac expression

- No rescue or no ectopic bristles

ND Not determined

Table 4. Summary of GFP expression in the transgenic reporter lines

Transgenic fly line	GFP expression in prepupal leg
ac promoter -*GFP*	+
ac-GFP4	+++
ac-GFP5	++++
ac-GFP-Act-H1*	+++++
ac-GFP-FL-H1*	++++
ac-GFP-FL-H1*H2*	++++
ac-GFP-FL-E*	+++
*a*c-GFP-FL-H1*H2*E*	+++++
ac-GFP-Act-H*+H	+++++
ac-GFP-Act-H*+E	+++++

+++++ Uniform GFP expression along the prepupal leg circumference

++++ Broad GFP expression excluding Hairy expression domain

+++ Narrow GFP expression as exhibited in wild type

+ GFP expression in a few cells

CREs, each of which activates *ac* expression in a discrete proneural field (Calleja et al., 2002). Alternatively, we find that a single activation element in the *ac*-mC-CRE can direct uniform expression throughout prepupal legs. This supports our suggestion that distinct mechanisms control *ac* expression in the MC vs mC proneural fields. Instead of the several modular CREs that activate expression in the MC proneural fields, *ac* expression in all the mC proneural fields can be activated by a single element.

In addition, the observation that *ac* expression in all the mC proneural fields is activated simultaneously by a single activation element within the *ac*-mC-CRE, is consistent with the suggestion that *ac/sc* expression is induced by a ubiquitously expressed transcriptional activator(s). However, we have not ruled out the possibility that expression is controlled by multiple locally acting factors that act through the *ac*-mC-CRE-Act.

4.6.2. Hairy acts through C-box sites and an E(spl)-like sequence to repress proneural *achaete* expression

Our observations suggest that Hairy acts directly through two C-box sequences, a previously identified site in the *ac* promoter and a second conserved, putative Hairy binding site in the *ac*-mC-CRE to repress *ac* expression. In addition we make the novel observation that Hairy can act through an E(spl)-like site. Previous studies have reported that Hairy preferentially binds sequences that contain the C-box core sequence, **CACGCG**, and that Hairy and E(spl) proteins have distinct DNA binding specificities (Bianchi-Frias et al., 2004; Jennings et al., 1999; Ohsako et al., 1994; Van Doren et al., 1994).

We assayed the function of a putative E(spl) binding site, TTG**CACGTG**CC, in the *ac*-mC-CRE that matches the consensus sequence, TGG**CACGTG**C/TC/TA, in 9/12

nucleotides. Several lines of evidence suggest that the E(spl) site is Hairy-responsive. We find, unexpectedly, that mutation of the H1, H2 and E(spl) sites in *ac*-mC-CRE transgenes result in more severe phenotypes than mutation of the just the H1 and H2 sites. For example, the triply mutant reporter and rescue transgenes exhibit higher levels of ectopic expression in the *hairy*-On interstripes than the H1/H2 mutant transgene. Furthermore, adults carrying the triply mutant rescue transgene, but not the H1/H2 mutant rescue transgene, in a wild type background exhibit phenotypes that are similar to *hairy* mutants, such as ectopic mC bristles in the wing blade and scutellum. Finally, we find that the *ac-GFP*-Act-H1*+E reporter gene is Hairy responsive. These observations suggest that, Hairy can act through an E(spl)-like, C/E-box, suggesting that there is more flexibility in its response sites than previously thought. This supposition is further supported by the observation that the triple mutant transgenes are responsive to overexpressed Hairy, although there is only one **CACGCG** sequence, the C-box core preferentially bound by Hairy, in the *ac*-mC-CRE.

4.6.3. N represses *achaete* expression through Hairy response sequences

Previous studies have shown that N repression of *ac* is mediated by genes of the E(spl) complex (Heitzler et al., 1996). The E(spl) proteins are bHLH repressors that have been shown to act through a palindromic sequence, TGG**CACGTG**C/TC/TA, which contains a C/E box core. As mentioned above, we identified one site with the E(spl) core, **CACGTG,** in the *ac*-mC-CRE. Therefore, we assayed the potential function of this site, TTG**CACGTG**CC, in mediating response to N signaling. However, two lines of evidence argue against a function for this site in mediating response to N signaling. First, mutational analysis of the putative E(spl) site has a mild or no effect on the activity of the *ac*-mC-CRE,

suggesting either that there are other redundant N response sites in the *ac*-mC-CRE or that the E(spl) site does not mediate response to N. Second the *ac-GFP*-Act-H1*+E reporter gene is not responsive to ectopic high-level N signaling.

On the other hand, our studies suggest that N can signal through the *ac* promoter H1 site and the *ac*-mC-CRE H2 site. This conclusion is supported by the finding that the triply mutant reporter and rescue transgenes exhibit expansion of *ac* or *GFP* expression into the *hairy*-Off interstripes. Since the E(spl) appears to be dispensable for response to N signaling, this would imply that N signals through one or both of the Hairy sites. In addition, we found the H1 site in the *ac* promoter can respond to high-level N signaling and that the *ac-GFP*-Act-H1*+E reporter is N responsive.

4.6.4. Common sequences mediate response to Hairy and N signaling.

Our investigations have provided novel insight into Hairy and N-mediated repression of proneural *ac* expression. We find that the response sites for these bHLH repressors are less specific than previously suggested. For example, Hairy represses *ac* expression via two sequences that contain a C-box core, CACGCG, which is consistent with previous observations. However, we present strong evidence suggesting that Hairy can also act through an E(spl)-like sequence that has a divergent core sequence, CACGTG. Furthermore, N appears to signal through the H1 and H2 sites instead of the E(spl)-like sequence.

Previous reports have suggested that Hairy and E(spl) proteins act through distinct target sequences (reviewed in Fisher and Caudy, 1998). We find, however, that Hairy and E(spl) can mediate repression of *ac* via common sequences in the *ac* promoter and *ac*-mC-CRE. This finding has interesting implications regarding the evolution of morphological

novelty in mC patterning. We suggest that the generation of novel mC patterns in specific regions of the leg or notum would likely be mediated by alteration of the spatially defined expression of the prepattern regulators of *ac* expression, Hairy and Dl. Indeed, we have observed that Hox genes function in the first and third leg to change the pattern of *Dl* expression, which results in an altered proneural prepattern (Shroff et al., 2007). This allows formation of novel mC patterns on these legs. Given that Hairy and N can act through common sites in the *ac*-mC-CRE, this would suggest that changes in these sequences would have a more global effect on mC patterning.

4.6.5. Model for regulation of *ac* expression in the mC proneural fields

Based on our findings, we propose the model shown in Figure 35 for regulation of *ac* expression in the mC proneural fields. We propose that one or more activators bind sequences in the *ac*-mC-CRE activation element to induce *ac* expression throughout prepupal legs. *ac* expression, however, is confined to the mC proneural fields because prior to induction of *ac* expression, a repressive prepattern is established via spatially defined expression of *hairy* and *Dl*. Hairy inhibits *ac* expression in the *hairy*-On interstripes through a Hairy binding site in the *ac* promoter, a putative Hairy binding site in the *ac*-mC-CRE and an E(spl)-like sequence in the *ac*-mC-CRE. Dl activates N signaling in the *hairy*-Off interstripes, which in turn turns on expression of one or more E(spl) proteins. E(spl) proteins repress *ac* expression via the two Hairy-binding sites. We note that Hairy and E(spl) also likely act through other sites in the *ac*-mC-CRE.

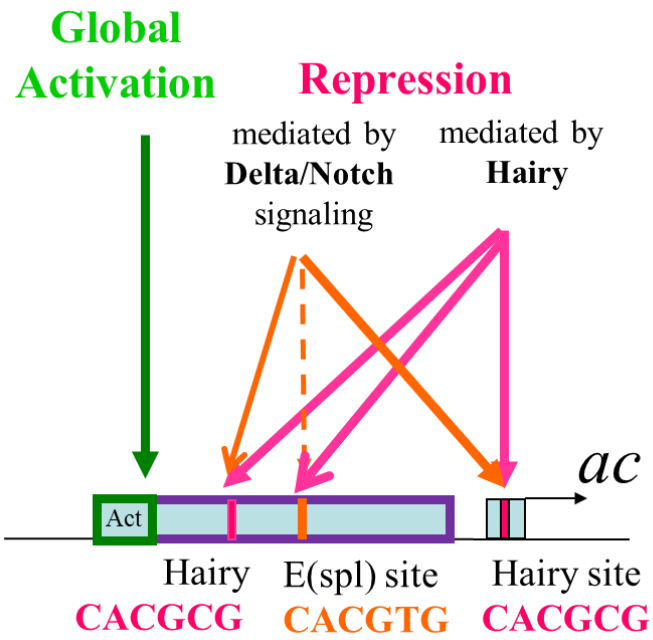


Figure 35. Model of mC patterning

Expression of *ac* in the primordia of late specified sensory organs, mCs, are controlled by a single CRE, which has one activation element and one repression element. In the absence of repressive influences, *ac* can be broadly expressed by unidentified activators through the activation element (box outlined in green). However, *ac* expression is spatially refined by repression mediated by Hairy and Dl/N signaling pathway through the repression element (box outlined in blue) and a Hairy site in the *ac* promoter. The Hairy site in the *ac* promoter can mediate repression by Hairy and Notch signaling. There is one potential Hairy site (pink bar), which can mediate repression by Hairy and Notch signaling, and one potential E(spl) site (orange bar) within the mC-CRE, which can mediate repression by Hairy.

CITED LITERATURE

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(2): 221-32.

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(16): 2003-13.

Artavanis-Tsakonas, S., Rand, M. D. and Lake, R. J. (1999) 'Notch signaling: cell fate control and signal integration in development', *Science* 284(5415): 770-6.

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(7): 1043-53.

Bae, Y. K., Shimizu, T. and Hibi, M. (2005) 'Patterning of proneuronal and inter-proneuronal domains by hairy- and enhancer of split-related genes in zebrafish neuroectoderm', *Development* 132(6): 1375-85.

Bailey, A. M. and Posakony, J. W. (1995) 'Suppressor of hairless directly activates transcription of enhancer of split complex genes in response to Notch receptor activity', *Genes Dev* 9(21): 2609-22.

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(10): 1167-81.

Basler, K. and Struhl, G. (1994) 'Compartment boundaries and the control of Drosophila limb pattern by hedgehog protein', *Nature* 368(6468): 208-14.

Bianchi-Frias, D., Orian, A., Delrow, J. J., Vazquez, J., Rosales-Nieves, A. E. and Parkhurst, S. M. (2004) 'Hairy transcriptional repression targets and cofactor recruitment in Drosophila', *PLoS Biol* 2(7): E178.

Bishop, S. A., Klein, T., Arias, A. M. and Couso, J. P. (1999) 'Composite signalling from Serrate and Delta establishes leg segments in Drosophila through Notch', *Development* 126(13): 2993-3003.

Brand, A. H. and Perrimon, N. (1993) 'Targeted gene expression as a means of altering cell fates and generating dominant phenotypes', *Development* 118(2): 401-15.

Bray, S. and Furriols, M. (2001) 'Notch pathway: making sense of suppressor of hairless', *Curr Biol* 11(6): R217-21.

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(5280): 1373-7.

Cabrera, C. V. and Alonso, M. C. (1991) 'Transcriptional activation by heterodimers of the achaete-scute and daughterless gene products of Drosophila', *EMBO J* 10(10): 2965-73.

Cabrera, C. V., Martinez-Arias, A. and Bate, M. (1987) 'The expression of three members of the achaete-scute gene complex correlates with neuroblast segregation in Drosophila', *Cell* 50(3): 425-33.

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(1-2): 1-12.

Campuzano, S. (2001) 'Emc, a negative HLH regulator with multiple functions in Drosophila development', *Oncogene* 20(58): 8299-307.

Campuzano, S. and Modolell, J. (1992) 'Patterning of the Drosophila nervous system: the achaete-scute gene complex', *Trends Genet* 8(6): 202-8.

Chen, Y. and Struhl, G. (1996) 'Dual roles for patched in sequestering and transducing Hedgehog', *Cell* 87(3): 553-63.

Cooper, M. T. and Bray, S. J. (1999) 'Frizzled regulation of Notch signalling polarizes cell fate in the Drosophila eye', *Nature* 397(6719): 526-30.

Cooper, M. T., Tyler, D. M., Furriols, M., Chalkiadaki, A., Delidakis, C. and Bray, S. (2000) 'Spatially restricted factors cooperate with notch in the regulation of Enhancer of split genes', *Dev Biol* 221(2): 390-403.

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(6): 996-1008.

Davis, R. L. and Turner, D. L. (2001) 'Vertebrate hairy and Enhancer of split related proteins: transcriptional repressors regulating cellular differentiation and embryonic patterning', *Oncogene* 20(58): 8342-57.

de Celis, J. F., de Celis, J., Ligoxygakis, P., Preiss, A., Delidakis, C. and Bray, S. (1996) 'Functional relationships between Notch, Su(H) and the bHLH genes of the E(spl) complex: the E(spl) genes mediate only a subset of Notch activities during imaginal development', *Development* 122(9): 2719-28.

de Celis, J. F., Tyler, D. M., de Celis, J. and Bray, S. J. (1998) 'Notch signalling mediates segmentation of the Drosophila leg', *Development* 125(23): 4617-26.

Delidakis, C. and Artavanis-Tsakonas, S. (1992) 'The Enhancer of split [E(spl)] locus of Drosophila encodes seven independent helix-loop-helix proteins', *Proc Natl Acad Sci U S A* 89(18): 8731-5.

Delidakis, C., Preiss, A., Hartley, D. A. and Artavanis-Tsakonas, S. (1991) 'Two genetically and molecularly distinct functions involved in early neurogenesis reside within the Enhancer of split locus of Drosophila melanogaster', *Genetics* 129(3): 803-23.

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(6502): 175-9.

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(5268): 1621-5.

Dominguez, M. and Campuzano, S. (1993) 'asense, a member of the Drosophila achaete-scute complex, is a proneural and neural differentiation gene', *EMBO J* 12(5): 2049-60.

Fichelson, P. and Gho, M. (2003) 'The glial cell undergoes apoptosis in the microchaete lineage of Drosophila', *Development* 130(1): 123-33.

Fisher, A. and Caudy, M. (1998) 'The function of hairy-related bHLH repressor proteins in cell fate decisions', *Bioessays* 20(4): 298-306.

Fortini, M. E. and Artavanis-Tsakonas, S. (1994) 'The suppressor of hairless protein participates in notch receptor signaling', *Cell* 79(2): 273-82.

Furriols, M. and Bray, S. (2001) 'A model Notch response element detects Suppressor of Hairless-dependent molecular switch', *Curr Biol* 11(1): 60-4.

Ghysen, A. and Dambly-Chaudiere, C. (1989) 'Genesis of the Drosophila peripheral nervous system', *Trends Genet* 5(8): 251-5.

Giagtzoglou, N., Alifragis, P., Koumbanakis, K. A. and Delidakis, C. (2003) 'Two modes of recruitment of E(spl) repressors onto target genes', *Development* 130(2): 259-70.

Gomez-Skarmeta, J. L., Campuzano, S. and Modolell, J. (2003) 'Half a century of neural prepatterning: the story of a few bristles and many genes', *Nat Rev Neurosci* 4(7): 587-98.

Gurdon, J. B. and Bourillot, P. Y. (2001) 'Morphogen gradient interpretation', *Nature* 413(6858): 797-803.

Hartenstein, V. and Posakony, J. W. (1990) 'A dual function of the Notch gene in Drosophila sensillum development', *Dev Biol* 142(1): 13-30.

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(13): 2891-9.

Heitzler, P., Bourouis, M., Ruel, L., Carteret, C. and Simpson, P. (1996) 'Genes of the Enhancer of split and achaete-scute complexes are required for a regulatory loop between Notch and Delta during lateral signalling in Drosophila', *Development* 122(1): 161-71.

Held, L. I. and Heup, M. A. (1996) 'Genetic mosaic analysis of decapentaplegic and wingless gene function in the Drosophila leg', *Dev Genes Evol* 206(3): 180-194.

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(2): 549-58.

Ingham, P. W., Pinchin, S. M., Howard, K. R. and Ish-Horowicz, D. (1985) 'Genetic Analysis of the Hairy Locus in DROSOPHILA MELANOGASTER', *Genetics* 111(3): 463-86.

Jarman, A. P., Brand, M., Jan, L. Y. and Jan, Y. N. (1993) 'The regulation and function of the helix-loop-helix gene, asense, in Drosophila neural precursors', *Development* 119(1): 19-29.

Jarriault, S., Brou, C., Logeat, F., Schroeter, E. H., Kopan, R. and Israel, A. (1995) 'Signalling downstream of activated mammalian Notch', *Nature* 377(6547): 355-8.

Jennings, B. H., Tyler, D. M. and Bray, S. J. (1999) 'Target specificities of Drosophila enhancer of split basic helix-loop-helix proteins', *Mol Cell Biol* 19(7): 4600-10.

Jiang, J. and Struhl, G. (1996) 'Complementary and mutually exclusive activities of decapentaplegic and wingless organize axial patterning during Drosophila leg development', *Cell* 86(3): 401-9.

Jimenez, F. and Campos-Ortega, J. A. (1990) 'Defective neuroblast commitment in mutants of the achaete-scute complex and adjacent genes of D. melanogaster', *Neuron* 5(1): 81-9.

Joshi, M., Buchanan, K. T., Shroff, S. and Orenic, T. V. (2006) 'Delta and Hairy establish a periodic prepattern that positions sensory bristles in Drosophila legs', *Dev Biol* 293(1): 64-76.

Kidd, S., Lieber, T. and Young, M. W. (1998) 'Ligand-induced cleavage and regulation of nuclear entry of Notch in Drosophila melanogaster embryos', *Genes Dev* 12(23): 3728-40.

Knust, E., Schrons, H., Grawe, F. and Campos-Ortega, J. A. (1992) 'Seven genes of the Enhancer of split complex of Drosophila melanogaster encode helix-loop-helix proteins', *Genetics* 132(2): 505-18.

Kojima, T. (2004) 'The mechanism of Drosophila leg development along the proximodistal axis', *Dev Growth Differ* 46(2): 115-29.

Kwon, C., Hays, R., Fetting, J. and Orenic, T. V. (2004) 'Opposing inputs by Hedgehog and Brinker define a stripe of hairy expression in the Drosophila leg imaginal disc', *Development* 131(11): 2681-92.

Lai, E. C. (2004) 'Notch signaling: control of cell communication and cell fate', *Development* 131(5): 965-73.

Lai, E. C. and Orgogozo, V. (2004) 'A hidden program in Drosophila peripheral neurogenesis revealed: fundamental principles underlying sensory organ diversity', *Dev Biol* 269(1): 1-17.

Lecourtois, M. and Schweisguth, F. (1995) 'The neurogenic suppressor of hairless DNA-binding protein mediates the transcriptional activation of the enhancer of split complex genes triggered by Notch signaling', *Genes Dev* 9(21): 2598-608.

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(6581): 387-93.

Lecuit, T. and Cohen, S. M. (1997) 'Proximal-distal axis formation in the Drosophila leg', *Nature* 388(6638): 139-45.

Marigo, V. and Tabin, C. J. (1996) 'Regulation of patched by sonic hedgehog in the developing neural tube', *Proc Natl Acad Sci U S A* 93(18): 9346-51.

Martinez, C., Modolell, J. and Garrell, J. (1993) 'Regulation of the proneural gene achaete by helix-loop-helix proteins', *Mol Cell Biol* 13(6): 3514-21.

Morata, G. (2001) 'How Drosophila appendages develop', *Nat Rev Mol Cell Biol* 2(2): 89-97.

Murre, C., McCaw, P. S. and Baltimore, D. (1989) 'A new DNA binding and dimerization motif in immunoglobulin enhancer binding, daughterless, MyoD, and myc proteins', *Cell* 56(5): 777-83.

Nellen, D., Burke, R., Struhl, G. and Basler, K. (1996) 'Direct and long-range action of a DPP morphogen gradient', *Cell* 85(3): 357-68.

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(22): 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(1): 9-20.

Parks, A. L., Huppert, S. S. and Muskavitch, M. A. (1997) 'The dynamics of neurogenic signalling underlying bristle development in Drosophila melanogaster', *Mech Dev* 63(1): 61-74.

Penton, A. and Hoffmann, F. M. (1996) 'Decapentaplegic restricts the domain of wingless during Drosophila limb patterning', *Nature* 382(6587): 162-4.

Posakony, J. W. (1994) 'Nature versus nurture: asymmetric cell divisions in Drosophila bristle development', *Cell* 76(3): 415-8.

Powell, L. M. and Jarman, A. P. (2008) 'Context dependence of proneural bHLH proteins', *Curr Opin Genet Dev* 18(5): 411-7.

Rauskolb, C. and Irvine, K. D. (1999) 'Notch-mediated segmentation and growth control of the Drosophila leg', *Dev Biol* 210(2): 339-50.

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(7): 997-1007.

Rubin, G. M. and Spradling, A. C. (1982) 'Genetic transformation of Drosophila with transposable element vectors', *Science* 218(4570): 348-53.

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(10): 3095-103.

Schweisguth, F. and Posakony, J. W. (1992) 'Suppressor of Hairless, the Drosophila homolog of the mouse recombination signal-binding protein gene, controls sensory organ cell fates', *Cell* 69(7): 1199-212.

Shroff, S., Joshi, M. and Orenic, T. V. (2007) 'Differential Delta expression underlies the diversity of sensory organ patterns among the legs of the Drosophila adult', *Mech Dev* 124(1): 43-58.

Simpson, P. (1990) 'Notch and the choice of cell fate in Drosophila neuroepithelium', *Trends Genet* 6(11): 343-5.

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(6): 984-95.

Tabata, T. (2001) 'Genetics of morphogen gradients', *Nat Rev Genet* 2(8): 620-30.

Tabata, T. and Kornberg, T. B. (1994) 'Hedgehog is a signaling protein with a key role in patterning Drosophila imaginal discs', *Cell* 76(1): 89-102.

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(12): 3939-48.

van den Heuvel, M. and Ingham, P. W. (1996) 'smoothened encodes a receptor-like serpentine protein required for hedgehog signalling', *Nature* 382(6591): 547-51.

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(22): 2729-42.

Van Doren, M., Ellis, H. M. and Posakony, J. W. (1991) 'The Drosophila extramacrochaetae protein antagonizes sequence-specific DNA binding by daughterless/achaete-scute protein complexes', *Development* 113(1): 245-55.

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 U S A* 94(6): 2404-9.

VITA

Ji Inn Lee

Laboratory for Molecular Biology
Department of Biological Sciences
University of Illinois at Chicago
Chicago, IL

EDUCATION

Current	Ph.D. Candidate Laboratory for Molecular Biology University of Illinois at Chicago, IL
2004	M.S., Biology New York University, New York, NY
2002	B.A., Biological Sciences State University of New York at Buffalo, NY
2000	B.S., Biology Kyung Hee University, Seoul, Korea

EMPLOYMENT AND EXPERIENCE

2004-2012	Teaching Assistant for Genetics Laboratory BIOS 221, UIC, IL Teaching Assistant for Microbiology Laboratory BIOS 351, UIC, IL
2002-2004	Master's Thesis Project, Developmental Genetics Lab, NYU, NY Advisor: Jane Hubbard, Ph.D.
2001-2002	Independent research in Molecular Genetics Lab, SUNY Buffalo, NY
1998-1999	Independent research in Neurobiology Lab, Kyung Hee University, Seoul, Korea Advisor: Yunhee Kim Kwon, Ph.D.

PRESENTATIONS

2012	Annual Drosophila Research Conference, Chicago, IL
2010	Society for Developmental Biology, Chicago, IL
2007-2010	Annual Drosophila Research Conference
2007-2009	Midwest Drosophila Conference, Monticello, IL

PUBLICATION

Maciejowski, J., Ahn, J.H., Cipriani. P.G., Killian, D.J., Chaudhary, A.L., Lee, J., Voutev, R., Johnsen, R.C., Baillie, D.L., Gunsalus, K.C., Fitch, D.A., and Hubbard, E.J. A (2005) Autosomal Genes of Autosomal/X-Linked Duplicated Gene Pairs and Germ-Line Proliferation in *Caenorhabditis elegans*, *Genetics* 169(4): 1997–2011