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Cell-cell signaling during *Drosophila* neurogenesis and wing development

by

Daniel Doherty

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Genetics

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA

San Francisco

This thesis dissertation is dedicated to my Mom and Dad, who made me who I am today.

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ABSTRACT

In the developing *Drosophila* nervous system, the neurogenic genes mediate a cell-cell communication process called lateral inhibition, in which a single cell adopts the neural cell fate and forces the surrounding cells to become neural. We demonstrate that the neurogenic gene *big brain* encodes a membrane protein that specifically rescues the neurogenic phenotype in *big brain* mutant embryos. big brain protein is expressed in tissues that give rise to neural precursors and in other tissues that are affected by loss of neurogenic gene function. Using mosaic analysis, we demonstrate that *big brain* activity is required autonomously in epidermal precursors to prevent neural development. Furthermore, we provide evidence that ectopically expressed bib acts synergistically with ectopically expressed Delta, causing cell fate transformations in adult sense organs. These results are consistent with bib acting as a channel protein required to respond to the lateral inhibition signal in cells which adopt the epidermal cell fate.

The neurogenic genes, and in particular *Notch* are also required for wing margin development. We demonstrate that Delta, a ligand for the Notch receptor, is essential for this process. *Delta* is required in ventral cells at the dorsal/ventral compartment boundary, where its expression is specifically elevated in second instar wing discs during wing margin formation. Moreover, ectopic Delta expression induces *wingless*, *vestigial* and *cut*, and causes adult wing tissue outgrowth in the dorsal compartment. Whereas ectopic expression of Notch induces *cut* in both dorsal and ventral compartments, ectopic Delta expression induces *cut* only in the dorsal compartment. Serrate, another Notch ligand, is required for wing development only in the dorsal compartment, and ectopic Serrate expression induces *cut* only in the ventral compartment. These observations indicate that *Notch* expressing cells in a given compartment have

different responses to Delta and Serrate. We propose that *Delta* and *Serrate* function as compartment-specific signals in the wing disc, activating *Notch* to induce downstream genes required for wing formation.

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CHAPTER 1

INTRODUCTION

Our understanding of development has benefited greatly from the study of *Drosophila melanogaster*. The unique combination of powerful genetics, molecular biology and ease of examining phenotypic variation and gene expression patterns has made this system particularly attractive for studying how cell fates are determined. Determination of fates within developing fields of cells is a process called pattern formation. Pattern formation can occur in both growing and non-growing fields of cells. However, much of what we have learned about pattern formation comes from experiments on the *Drosophila* embryo, where there is cell division but no growth. In contrast, many developmental processes occur in growing tissues such as organs and appendages, including *Drosophila* legs, wings, and halteres. It is therefore possible to take full advantage of the genetic and molecular techniques available in *Drosophila* to address the general problem of how both growing and non-growing tissues are patterned.

There are two major classes of mechanisms that control the determination of cell fates, intrinsic and extrinsic (reviewed in Slack, 1991). Cell intrinsic mechanisms are independent of the environment surrounding a cell, whereas cell extrinsic mechanisms require communication with the environment. In this thesis, I will describe two examples where cell extrinsic mechanisms play an important role during the development of *Drosophila*. Chapter 2 describes the role of the *big brain* gene in lateral inhibition, a cell-cell communication process required for restricting the number of neural precursor cells in non-growing fields of cells. Chapter 3 describes the role of the *Dl* gene in cell-cell communication at the dorsal/ventral (D/V) boundary required for growth and patterning of the wing.

Cell-cell communication

Determination of cell fates by cell-cell communication mechanisms involves the production of a signal by one cell which is received by an adjacent cell. The signal is transmitted to the nucleus effecting changes in patterns of gene expression which modify the fate of the receiving cell. A number of different signaling pathways play important roles during *Drosophila* development. These pathways can function in several contexts; for example, the *N* signaling pathway functions in lateral inhibition during neurogenesis, in transcompartmental signaling across the (D/V) boundary during wing development as well as in many other processes (reviewed in Artavanis-Tsakonas *et al.*, 1995). To ensure that each pathway functions only in the appropriate context, the ability of cells to send a signal as well as their ability to respond to a signal can be modulated. In addition, more than one signaling pathway can play a role in patterning of the same tissue; multiple signals can be integrated within a single cell to determine its final fate. In Chapter 4, I will discuss the questions raised by the work presented in Chapters 2 and 3. I will also discuss how *N* pathway activity is modulated in different cell populations and integrated with other signaling pathways during *Drosophila* embryonic and imaginal development.

The neurogenic genes

In *Drosophila*, single neural precursors (NP's) segregate from groups of competent cells called proneural clusters. The NP prevents additional proneural cells from adopting the neural fate through a process called lateral inhibition (see Chapter 2). Loss of function mutations in a group of genes - *Notch* (*N*), *Delta* (*Dl*), *Suppressor of Hairless* (*Su(H)*), *Enhancer of split* (*E(spl)*), *mastermind* (*mam*), *neuralized* (*neu*), and *big brain* (*bib*) - cause striking hypertrophy of the nervous system; this hypertrophy is commonly referred to as the neurogenic phenotype.

In addition to nervous system development, many of these genes are required in a variety of other developmental contexts, including formation of the wing margin, somatic muscle, Malpighian tubules, and ventral midline cells of the embryonic central nervous system (Corbin et al., 1991; Hartenstein et al., 1992; Hoch et al., 1994; Lindsley and Zimm, 1992; Menne and Klambt, 1994). A number of these genes are also required to generate anterior/posterior polarity in the oocyte and for axon guidance (Giniger et al., 1993; Ruohola et al., 1991).

The shared neurogenic phenotype resulting from mutations in these genes indicates that they all function in the process of lateral inhibition. Interactions between all of these genes, except *bib*, implicate them in a single signaling pathway, which I will refer to as the *N* pathway for the purposes of this thesis. A number of different strategies have been used to demonstrate interactions involving the neurogenic genes. One type of study has shown that raising or lowering the copy number of one neurogenic gene can alter the loss of function phenotype of another neurogenic gene (de la Concha et al., 1988; Vässin et al., 1985). de la Concha et al. (1988) proposed a genetic pathway based on the premise that decreasing the copy number of downstream genes would modulate the phenotype of upstream genes, but not vice versa; however, these genetic interactions are quite complex. Transheterozygous combinations between loss of function *E(spl)* alleles and either loss of function *N* alleles or loss of function *Dl* alleles cause embryonic lethality. Whereas, transheterozygous combination between loss of function *N* alleles and loss of function *Dl* alleles not only does not cause embryonic lethality, and in fact, the adult $N^-/+$; $Dl^-/+$ flies have reduced *Dl* and *N* haploinsufficient wing phenotypes. Another type of study used the recessive viable *N* allele, *split*, to isolate mutations that enhance (*Hairless*, *mam*, *daughterless*, *roughened eye*, and *glass*) and suppress (*Dl* and *scabrous*) the *split* rough eye phenotype in a dominant manner (Brand and Campos-Ortega, 1989).

A third type of study used a novel lethal combination of dominant *N* alleles to isolate mutations in *Dl* and *mam* that suppress the lethality in a dominant manner (Xu et al., 1990). These studies have identified genes that are candidates to function in the *N* signaling pathway and because of allele-specific interactions it has been suggested that some of the gene products may interact physically. The full significance of these genetic interactions is becoming clear only in light of further molecular and biochemical data.

Isolation and molecular analysis of the neurogenic genes (Table 1.1) has led to a model for how the *N* signaling pathway functions. Using mosaic analysis in *Drosophila*, Heitzler and Simpson (1991; 1993) demonstrated that *N* acts cell autonomously to receive the lateral inhibition signal, while *Dl* acts non-autonomously and is required to send the signal. Fehon *et al.* (1990) demonstrated in cultured S2 cells that Dl and N proteins on separate cells bind to each other. Fortini and Artavanis-Tsakonas (1994) found that this binding event allows the Su(H) protein to move from the cytoplasm to the nucleus in the N-expressing cells, although this change in subcellular localization has not been observed in vivo. In addition, there may be other consequences of N activation independent of *Su(H)*. Homologues of *N, Dl, E(spl)*, and *Su(H)* have been identified in mammals (Furukawa *et al.*, 1992; Sasai *et al.*, 1992; Schweisguth and Posakony, 1992; Lindsell *et al.*, 1995; and reviewed in Artavanis-Tsakonas *et al.*, 1995). More recent in vivo studies in *Drosophila* and in vitro studies in mammalian tissue culture have shown that Su(H) activates transcription of the *E(spl)*-C genes in a *N*-dependent manner, thus indicating that this mechanism is conserved between *Drosophila* and vertebrates (Bailey and Posakony, 1995; Jarriault et al., 1995; Lecourtois and Schweisguth, 1995). In addition, at least part of the N protein may be translocated to the nucleus, where it may act in concert with Su(H) (Jarriault et al., 1995; Lieber et al., 1993; Struhl et al., 1993). It has been

shown that the bHLH transcription factors encoded by the *E(spl)-C* form a complex with groucho, a nuclear protein with repeated WD40 motifs, to regulate transcription of downstream genes responsible for executing cell fates (Paroush et al., 1994; van der Voorn and Ploegh, 1992). As yet, no specific functions have been ascribed to *mam* and *neu*, but the neurogenic phenotype caused by loss of function alleles of both genes can be modified by changes in the copy number of the other neurogenic genes (de la Concha et al., 1988). In contrast, *bib* has not been found to interact with other genes under any conditions.

N pathway activation in the receiving cells appears to decrease their capacity to send the signal, creating an intercellular feedback loop which reinforces signaling in one cell and receiving in the other cells. This feedback loop is most apparent during *C. elegans* vulval development, where the *N*-like gene *lin-12* and the gene that encodes its presumptive ligand, *lag-2*, are required for proper formation of the ventral uterine precursor cell (VU) and the anchor cell (AC). In the absence of *lin-12* or *lag-2* activity, two AC cells are formed. Both genes are initially expressed in the two cells that will become the VU and AC; however, prior to commitment of these cells, *lin-12* becomes restricted to the presumptive VU and *lag-2* becomes restricted to the presumptive AC. It appears that *lin-12* mediates this process, because *lin-12* activity can enhance its own expression and repress *lag-2* expression (Wilkinson et al., 1994).

The big brain gene

The fact that *bib* alleles have not been found to interact with mutations in any of the other neurogenic genes may be a function of the methods used to look for genetic interactions as well as the particular genetic characteristics of *bib*. The phenotypes of different *bib* alleles are indistinguishable, and in the absence of a deficiency for the *bib* genomic region, it is not possible to determine whether the

known alleles are genetic nulls. Most genetic strategies for isolating interacting genes have made use of viable allele combinations. Since there are no viable *bib* alleles, these types of methods have not been possible. The most comprehensive genetic study of interactions between the neurogenic genes relied on the observation that removing a copy of one neurogenic gene often modifies the homozygous null neurogenic phenotype of another gene, a dominant haploinsufficient interaction (de la Concha et al., 1988). Genes like *bib* that show no dosage sensitivity might not have an effect in this type of assay. The lack of viable *bib* alleles and lack of dosage sensitivity has made it difficult to assess whether *bib* functions in the *N* pathway.

bib was isolated by Rao *et al.* (1990) and found to encode a 701 amino acid protein with sequence similarity to a family of small molecule channel proteins (Figure 1.1). This family is characterized by 20-50% amino acid identity, six hydrophobic domains, and two conserved stretches of 10-15 amino acids (>70% identity) at characteristic positions between the second and third and between the fifth and sixth hydrophobic domains (Reizer et al., 1993). These channel proteins are present in organisms as diverse as bacteria and vertebrates, and while some of them have been shown to transport small molecules like water and glycerol across cell membranes (Chrispeels and Agre, 1994), none of them have been shown to affect cell fates. Therefore, the study of *bib* may offer an opportunity to discover novel mechanisms for cell fate determination involving channel-like proteins.

It has been assumed that *bib* functions in parallel to the other neurogenic genes (reviewed in Campos-Ortega and Jan, 1991). In the second chapter of this thesis I will present several lines of evidence that suggest that bib does function in the *N* signaling pathway. *bib* is expressed in the same tissues that require neurogenic gene activity, and bib protein colocalizes with Dl and N, indicating

that it functions in physical proximity to the other neurogenic genes. Furthermore, *bib* is required for the proper reception of the lateral inhibition signal, and we have preliminary evidence that ectopically expressed bib functions synergistically with ectopically expressed Dl.

Neurogenic gene function in wing development

We have examined the role of the *N* pathway in patterning a growing tissue, the *Drosophila* wing. The wing develops from a cluster of cells, the wing imaginal disc, that is set aside in the embryo. Initially, the disc contains 20-40 undifferentiated cells and little pattern information. By the end of the third larval instar, the disc has proliferated and developed the complex pattern elements of the mature wing. In addition to growth and patterning during larval development, the disc undergoes radical morphological changes during pupal development. The dorsal cells become apposed to the ventral cells as the disc folds and everts along the dorsal/ventral (D/V) boundary to form the mature wing blade which is comprised of two symmetrical cell layers which adhere to each other. The D/V boundary becomes the wing margin, marked with sensory bristles along the anterior margin and large non-innervated hairs along the posterior margin.

The wing consists of four compartments (anterior, posterior, dorsal and ventral) as defined by lineage restriction studies (Blair, 1993; Garcia-Bellido et al., 1973). Excision of tissue from developing appendages induces proliferation and intercalation of pattern elements (for review see French et al., 1976). More recently, it has been proposed that interaction between cells with different compartmental identities generates organizing centers which govern the growth and patterning of the surrounding tissues (Diaz-Benjumea and Cohen, 1993; Diaz-Benjumea and Cohen, 1995; Meinhardt, 1983; Tabata and Kornberg, 1994).

Some of the molecular events that generate the organizing center at the anterior/posterior (A/P) boundary have recently been deciphered. Expression of the engrailed (en) homeodomain protein defines posterior compartment cells, induces expression of the secreted hedgehog (hh) protein, and makes posterior cells refractory to hh signaling. This mechanism ensures that hh induces the transforming growth factor ß family member, *decapentaplegic* (*dpp*), only in the cells anterior to the compartment boundary (Basler and Struhl, 1994; Tabata and Kornberg, 1994). It has been proposed that *dpp* is responsible for much of the organizing function of the A/P boundary (Tabata et al., 1995; Zecca et al., 1995). The asymmetric boundary between *en* -expressing and non-expressing cells generates different patterns of growth, gene expression and adult cuticle structures in the anterior and posterior, resulting in the asymmetric A/P axis of the adult wing.

The requirements for patterning at the dorsal/ventral compartment boundary are significantly different from the requirements at the A/P boundary . While the A/P axis is quite asymmetric, the sheets of dorsal and ventral cells must be symmetrical with respect to size and vein pattern, so that they can adhere to each other to form the adult wing. The signaling events that generate symmetrical patterns of growth and gene expression from the asymmetric D/V boundary are not as well characterized, but a number of the genes involved have been identified. Dorsal but not ventral cells express *apterous* (*ap*), a homeodomain transcription factor which is required for wing disc proliferation and formation of the wing margin (Bourgouin et al., 1992; Diaz-Benjumea and Cohen, 1993; Williams et al., 1993). The function of ap is analogous to that of en; ap induces the expression of *fringe* (*fng*), which encodes a novel, putatively secreted molecule (Irvine and Wieschaus, 1994). Juxtaposition of *fng* expressing and *fng* non-expressing cells induces tissue outgrowth and the formation of wing

margin structures, suggesting that *fng* encodes a signal that can only be received by cells that do not express *fng*. To explain that both *fng*⁻ and *fng*⁺ cells at the borders of *fng*⁻ clones are transformed into wing margin, Irvine and Wieschaus (1994) have proposed that *fng* is a dorsal to ventral signaling molecule that induces a reciprocal (ventral to dorsal) signal from the *fng*⁻ cells to the *fng*⁺ cells. The existence of a reciprocal signal has also been proposed by Williams et al. (1994) to explain the similar behavior of *ap* clones.

Couso et al.(1995) have proposed that *wingless* (*wg*) is a ventral to dorsal signal required for wing development. In second instar wing discs, *wg* is expressed in the ventral compartment, and may function to maintain the restriction of *ap* expression to the dorsal compartment (Williams et al., 1993). However, loss of wg function in wing margin cells on both sides of the D/V boundary causes loss of wing margin structures and loss of the wing blade, while loss of wg on only one side of the boundary or within the blade disrupts neither the wing margin nor the wing blade (Diaz-Benjumea and Cohen, 1995). This indicates that wg is required at the D/V boundary to organize the wing, but it cannot be a ventral to dorsal signal because it is not required specifically in the ventral compartment.

The *N* signaling pathway is required for signaling across the D/V boundary (de Celis and Garcia-Bellido, 1994; Rulifson and Blair, 1995). Loss of function mutations in *N* cause loss of wing tissue similar to that observed in *wg* mutants, and genetic interactions between *N* and *wg* indicate that the two genes function in the same pathway during wing margin formation (Couso and Martinez Arias, 1994; Hing et al., 1994). Couso and Martinez Arias (1994) have proposed models in which *wg* acts upstream or parallel to *N* and may even be a N ligand during wing margin development ; however, more recent studies by Rulifson and Blair (1995) have shown that *wg* expression at the wing margin

requires *N* activity, suggesting that *N* functions upstream of *wg*. Loss of *N* in cells on either side of the D/V boundary causes loss of *wg* expression, as well as loss of wing margin and blade tissue in both compartments. This highlights an unusual situation. Cells on each side of the boundary depend on the *N* activity in cells on the other side of the boundary; therefore, *N* signaling is required in both directions across the D/V boundary for wing formation.

Several observations led Kim et al. (1995) and Diaz-Benjumea and Cohen (1995) to propose that *Serrate* (*Ser*), which encodes another N ligand with sequence similarity to Dl (Fleming et al., 1990; Rebay et al., 1991), functions downstream of *fng* and acts as a dorsal to ventral signal. *Ser* expression is restricted to the dorsal compartment in mid-second instar discs during the early steps of wing margin formation (Diaz-Benjumea and Cohen, 1995). Loss of *Ser* function in the dorsal compartment results in loss of the wing margin and adjacent blade tissue, whereas loss of *Ser* function in the ventral compartment has no effect (Diaz-Benjumea and Cohen, 1995). Ectopic expression of *Ser* in both the dorsal and ventral compartments induces adult wing tissue outgrowth and *wg* expression only in the ventral compartment (Speicher, et al., 1994; Kim et al., 1995; Diaz-Benjumea and Cohen, 1995). The fact that ectopic *fng* expression induces Ser, but ectopic *Ser* does not induce *fng* places *Ser* downstream of *fng* (Kim, et al., 1995). The properties of *Ser* make it a good candidate for a dorsal to ventral, N-dependent signal, but do not account for the ventral to dorsal N-dependent signal.

In Chapter 3 of this thesis, we provide strong evidence that Dl, the other known N ligand, is a ventral to dorsal ligand required for wing formation. The mild wing notching observed with temperature sensitive combinations of *Dl* alleles has implicated *Dl* in wing development, although its role has remained unclear (Parody and Muskavitch, 1993). Using clonal analysis, we demonstrate

that *Dl* is required specifically in the ventral compartment for wing development. Ectopically expressed Dl can induce wing outgrowth and expression of genes required for wing formation including *wg*, *vg* and *cut*. Furthermore, dorsal cells are more receptive to ectopic *Dl* signal than ventral cells, as indicated by the restriction of Dl-induced *cut* expression and adult wing outgrowth to the dorsal compartment. In contrast, dorsal cells do not respond to ectopic *Ser*, while ventral cells do, indicating that *N* expressing cells can have different responses to the two *N* ligands. The specific responses to Dl and Ser in the dorsal and ventral compartment require a unique mechanism for modulating the *N* pathway response in a position-specific manner. We propose that *N*, *Dl* and *Ser* are components of a positive feedback loop across the D/V boundary that generates the precisely symmetrical patterns of growth and gene expression required to form the mirror image sheets of dorsal and ventral cells in the adult wing.

Table 1.1 Predicted products of the neurogenic genes

gene	gene product	reference
Delta	transmembrane domain, EGF-like repeats	Vässin et al. (1987)
Notch	transmembrane domain, EGF-like repeats, CDC10/ankryn repeats	Wharton et al. (1985), Kidd et al. (1986)
Supressor of Hairless	N binding, transcriptional activator	Schweisguth and Posakony (1992)
Enhancer of split	bHLH transcriptional regulators	Klämbt et al. (1989) Knust et al. (1992)
groucho	nuclear protein, WD40 repeats	Hartley et al. (1988)
mastermind	nuclear protein	Smoller et al. (1990)
neuralized	cytoplasmic protein, zinc finger	Boulianne et al. (1991) Price, et al. (1993) G. Feger, personal communication
big brain	similarity to channel proteins	Rao et al. (1990)
shaggy	serine-threonine kinase	Bourouis et al. (1990)

gene | activity

bib (*Drosophila*) N — C unknown

CHIP28 (vertebrate) N — C water channel

MIP (vertebrate) N — C unknown

GlpF (E. coli) N — C glycerol channel

FPS (yeast) N — C unknown

20-50% identity overall

Figure 1.1 *bib* shares sequence similarity with the MIP family of genes.

MIP family members encode proteins with six hydrophobic domains (black boxes) and two highly conserved stretches of 10-15 amino acids, each centered on the invariant asparagine-proline-alanine sequence (white boxes). The amino acid identity between family members over the entire channel domain is 20-50% (Reizer et al., 1993). Family members have been isolated from bacteria, yeast, plants and vertebrates. It has been demonstrated that some of these proteins exhibit water and glycerol transport activity (reviewed in Chrispeels and Agre, 1994).

```
                           10        20        30        40        50        60
                  . . . . . . . . | . . . . . . . . . | . . . . . . . . . | . . . . .
                   4 4  2        4
MIP(Bta)    14    CAEFFASLFYVFFGLGASL..RWAP.....GPLHV...........................LQ.
MIP(Hsa)    14    FAEFFATLFYVFFGLGSSL..RWAP.....GPLHV...........................LQ.
MIP(Rno)    X+12  FAEFFATLFYVFFGLGSSL..RWAP.....GPLHV...........................LQ.
ChIP(Hsa)   15    VAEFLATTLFVFISRGSALGFKYPV.....GNNQTA......................VQDNVK.
RtTIP(Nto)  22    VAEFIATLLFVFAGVGSAIAYNKLT.....ADAALD......................PAGLVA.
γTIP(Ath)   24    LAEFISTLIFVVAGSGSGMAFNKLT.....ENGATT......................PSGLVA.
αTIP(Ath)   27    LAEFLSTFVFVFAAEGSILSLDKLYWEHAAHAGTNT......................PGGLIL.
αTIP(Pvu)   24    LAEFASTFIFVFAGEGSGLALVKIY.....QDSAFS......................AGELLA.
WTIP(Ath)   40    IAEFVATLLFLYVTVLTVLIIYKIQ.SDTKAGGVDC......................GGVGIL.
WTIP(Psa)   58    IAEFIATFLFLYITVLTVMGVVRES.SKCKTVGIQ..........................GI..
BIB(Dme)    69    ISFCLASFMYVFIVCGAAAGVGVGA.....SVSSV..........................LLA.
NOD(Gma)    41    VAEAVGTYFLIFAGCASLVVNENYY.....NMITF...........................PG.
GLP(Eco)    12    IAEFLGTGLLIFFGVG.CVAALKVAG.....A.SFGQ.........................WE..
GLP(Bsu)    5     WGFVIGTMLLIIFGAGVCAGVNLKK.....SLSFQS........................GWIV.
ORF(Lla)    10    ITEFVGTALLIIMGNGAVANVELKG.....TKAHAQ........................SWMI.
GLP(Sco)    8     IGETIGTALLILLGGGVCAAVTLKA.....SKARNA........................GWLA.
FPS(Sce)    257   LAEFMGTMVMIIFGSAVVCQVNVAG.....KIQQDNFNVALDNLNVTGSSAETIDAMKSLTSLVS

Consensus          AEF  T   VF G G

                      70        80        90        100       110       120       130
                  . . . | . . . . . . . . . | . . . . . . . . . | . . . . . . . . . |
                            4                  411 1   13                 1  3
MIP(Bta)    44    .........VALAFGLALATLVQAVGH..ISGAHVNPAVTFAFLVGSQMSLLRAICYMVAQLLGA
MIP(Hsa)    44    .........VAMAFGLALATLVQSVGH..ISGAHVNPAVTFAFLVGSQMSLLRAFCYMAAQLLGA
MIP(Rno)    X+42  .........VALAFGLALATLVQTVGH..ISGAHVNPAVTFAFLVGSQMSLLRAFCYIAAQLLGA
ChIP(Hsa)   52    .........VSLAFGLSIATLAQSVGH..ISGAHLNPAVTLGLLLSCQISIFRALMYIIAQCVGA
RtTIP(Nto)  57    .........VAVAHAFALFVGVSIAAN..ISGGHLNPAVTLGLAVGGNITILTGFFYWIAQLLGS
γTIP(Ath)   61    .........AAVAHAFGLFVAVSVGAN..ISGGHVNPAVTFGAFIGGNITLLRGILYWIAQLLGS
αTIP(Ath)   69    .........VALAHAFALFAAVSAAIN..VSGGHVNPAVTFGALVGGRVTAIRAIYYWIAQLLGA
αTIP(Pvu)   59    .........LALAHAFALFAAVSASMH..VSGGHVNPAVTFGALIGGRISVIRAVYYWIAQLLGS
WTIP(Ath)   81    .........GAWAFGGMIFILVYCTAG..ISGGHINPAVTFGLFLTRKVSLIRAVLYMVAQCLGA
WTIP(Psa)   94    ..........AWAFGGMIFALVYCTAG..ISGGHINPAVTFGLFLARK.SLTRAIFYMVMQVLGA
BIB(Dme)    102   .........TALASGLAMATLTQCFLH..ISGAHINPAVTLALCVVRSISPIRAAMYITAQCGGG
NOD(Gma)    73    .........IAIVWGLVLTVLVYTVGH..ISGGHFNPAVTIAFASTRRFPLIQVPAYVVAQLLGS
GLP(Eco)    44    .........ISVIWGLGVAMAIYLTAG..VSGAHLNPAVTIALWLFACFDKRKVIPYIVSQVAGA
GLP(Bsu)    40    .........VVFGWGLGVAMAAYAVGG..ISGAHLNPALTIALAFVGDFPWKEVPVYIAAQMIGA
ORF(Lla)    45    .........IGWGYGLGVMLPAVAFGN..IT.SQTNPAFTLGLAASGLFPWAHVAQYIIAQVLGA
GLP(Sco)    43    .........IAFGWGFAVMTAAYISGP..LSGAHLNPAVTVGIAI
FPS(Sce)    317   SVAGGTFDDVALGWAAAVVMGYFCAGGSAISGAHLNPSITLANLVYRGFPLKKVPYYFAGSLIGA

Consensus                   A A GLA    V   G   ISGAH NPAVT G          RA  Y  AQ LGA

                           140       150       160       170       180       190
                  . . . . . | . . . . . . . . . | . . . . . . . . . | . . . . . . . .
MIP(Bta)    98    VAGAAVLYSVTPPAVRGN................LALNT..LHPGV.........SVGQATIVEI
MIP(Hsa)    98    VAGAAVLYSVTPPAVRGN................LALNT..LHPAV.........SVGQATTVEI
MIP(Rno)    X+96  VAGAAVLYSVTPPAVRGN................LALNT..LHAGV.........SVGQATTVEI
ChIP(Hsa)   106   IVATAILSGITSSLTGNS................LGRND..LADGV.........NSGQGLGIEI
RtTIP(Nto)  113   TVACLLLKYVTNGLAVPT................H...G..VAAGL.........NGLQGVVMEI
γTIP(Ath)   115   VVACLILKFATGGLAVPP................F...G..LSAGV.........GVLNAFVFEI
αTIP(Ath)   123   ILACLLLRLTTNGMRPVG................F...R..LASGV.........GAVNGLVLEI
αTIP(Pvu)   115   IVAALVLRLVTNNMRPSG................F...H..VSPGV.........GVGHMFILEV
WTIP(Ath)   135   ICGVGFVKAFQSSH.YVN................YGGGANFLADGY.........NTGTGLAAEI
WTIP(Psa)   147   ICGAGVVKGFEGKQRFGD................LNGGANFVAPGY.........TKGDGLGAEI
BIB(Dme)    156   IAGAALLYGVTVPGYQGN................LQ.AAISHSAAL.........AAWERFGVEF
NOD(Gma)    127   ILASGTL.RLLFMGNHDQ................FS.GTVPNGTNL.........QA...FVFEF
GLP(Eco)    98    FCAAALVYGLYYNLFFDFEQTHHIVRGSVESVDLAGTFSTYPNPHI.........NFVQAFAVEM
GLP(Bsu)    94    IIGAVIIY.LHYLPHWKSTDD....PAAK.....LGVFSTGPSIPH.........TF.ANVLSEV
ORF(Lla)    98    MFGQLLIV.MVYRPYYLKTQN....PNAI.....LGTFSTIDNVDDNSEKTRLGATI.NGFLNEF
FPS(Sce)    382   FTGALILF.IWYKRVLQEAYSDWWMNESV.....AGMFCVFPKPYL.........SSGRQFFSEF

Consensus           A   L   T                                 G                  EI
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                         200       210       220       230       240       250       260
                      ....|.........|.........|.........|.........|.........|.........|
                        2  4                             1             2           4  1
MIP(Bta)         136  FLTLQFVLCIFA....................TY.DERRNGR...LGSVA...........LAVG
MIP(Hsa)         136  FLTLQFVLCIFA....................TY.DERRNGQ...LGSVA...........LAVG
MIP(Rno)       X+134  FLTLQFVLCIFA....................TY.DERRNGR...MGSVA...........LAVG
MIP(Gga)         X+1                                     DRHD.GR...PGSAA...........LPVG
ChIP(Hsa)        144  IGTLQLVLCVLA....................TT.DRRRRD....LGGSAP..........LAIG
RtTIP(Nto)       148  IITFALVYTVYA....................TAADPKKGS....LGTIAP..........IAIG
γTIP(Ath)        150  VMTFGLVYTVYA....................TAIDPKNGS....LGTIAP..........IAIG
αTIP(Ath)        158  ILTFGLVYVVYS....................TLIDPKRGS....LGIIAP..........LAIG
αTIP(Pvu)        150  VMTFGLMYTVYG....................TAIDPKRGA....VSNIAP..........LAIG
WTIP(Ath)        174  IGTFVLVYTVFS....................AT.DPKRNARDSHVPVLAP..........LPIG
WTIP(Psa)        187  VGTFILVYTVFS....................AT.DAKRSARDSHVPILAP..........LPIG
BIB(Dme)         195  ILTSLVVLCYFV....................ST.DPMKKF....MGNSA...........ASIG
NOD(Gma)         162  IMTFFLMFVICG....................VATD.NRA.....VGELAG..........IAIG
GLP(Eco)         154  VITAILMGLILA....................LTVDGNGVPR....GPLAP..........LLIG
GLP(Bsu)         139  IGTFVLVLGILA....................IGAN.QFTE.....G.LNP..........LIVG
ORF(Lla)         152  LGSFVLPFGAVAATNIFFGSQSITWMTNYLKGQGADVSSSDVMNQIWVQASGASASKMIAHLPLG
FPS(Sce)         432  LCGAMLQAGTFA....................LT.DPYTCLSSD.VF...P..........LMMF

Consensus               TF LV    A                       D  R       G  AP          LAIG

                                270       280       290       300       310       320       330
                      .........|.........|.........|.........|.........|.........|.........|.
                                    1    1 2  43                        1    2   1       1
MIP(Bta)         166  FSLTLGHLFGMYYTGAGMNPARSFAPAIL..............TRNFTNHWVYWVGPVIGAGLGSLLYDFL
MIP(Hsa)         166  FSLALGHLFGMYYTGAGMNPARSFAPAIL..............TGNFTNHWVYWVGPIIGGGLGSLLYDFL
MIP(Rno)       X+164  FSLTLGHLFGMYYTGAGMNPARSFAPAIL..............TRNFSNHWVYWVGPIIGGGLGSLLYDFL
MIP(Gga)        X+15  FSLALGHLFGIPFTGAGMNPARSFAPAVI..............TRNFTNHWVFWAGPLLGAALAALLYELA
ChIP(Hsa)        174  LSVALGHLLAIDYTGCGINPARSFGSAVI..............THNFSNHWIFWVGPFIGGALAVLIYDFI
RtTIP(Nto)       179  FIVGANILAAGPFSGGSMNPARSFGPAVV..............AGDFSQNWIYWAGPLIGGGLAGFIYGDV
γTIP(Ath)        181  FIVGANILAGGAFSGASMNPAVAFGPAVV..............SWTWTNHWVYWAGPLVGGGIAGLIYEVF
αTIP(Ath)        189  LIVGANILVGGPFSGASMNPARAFGPALV..............GWRWHDHWIYWVGPFIGSALAALIYEYM
αTIP(Pvu)        181  LIVGANILVGGPFDGACMNPALAFGPSLV..............GWQWHQHWIFWVGPLLGAALAALVYEYA
WTIP(Ath)        208  FAVFMVHLATIPITGTGINPARSFGAAVI...........FNKSKPWDDHWIFWVGPFIGATIAAF.YHQF
WTIP(Psa)        221  FAVFLVHLATIPITGTGINPARSLGAAIV...........FNKKIGWNDHWIFWVGPFIGAALAAL.YHQV
BIB(Dme)         224  CAYSACCFVSMPY....LNPARSLGPSFV..............LNKWDSHWVYWFGPLVGGMASGLVYEYI
NOD(Gma)         191  STLLLNVIIGGPVTGASMNPARSLGPAFV..............HGEYEGIWIYLLAPVVGAIAGAWVYNIV
GLP(Eco)         185  LLIAVIGASMGPLTGFAMNPARDFGPKVFAWLAGWGNV.AFTGGRDIPYFLVPLFGPIVGAIVGAFAYRKL
GLP(Bsu)         167  FLIVAIGISLGGTTGYAINPARDLGPRIAHAFLPIP....GKGSSNWKYAWVPVVGPILGGSFGGVFYNAA
ORF(Lla)         217  FLVMGLVVALGGPTGPGLNPARDFGPRLVHSLLPKSVLGEAKGSSKWWYAWVPVLAPIL.ASLAAVALFKM
FPS(Sce)         462  ILIFIINASMAYQTGTAMNLARDLGPRLALYAVGFDHK.MLW.VHHHHFFWVPMVGPFIGALMGGLVYDVC

Consensus             F      L     TG  MNPARSFGP                       HWV WVGP LGA LA L Y
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Figure 1.2 Multiple alignment of 18 proteins of the MIP family. Numbers at the top of the aligned sequences denote the residue position in the multiple alignment. The residue number in each protein is provided at the beginning of each line. Positions that are fully conserved or that are conserved with only one exception are highlighted in black. The consensus sequence is provided below the multiple alignment (adapted from Reizer et al., 1993).

CHAPTER 2

The *Drosophila* neurogenic gene *big brain* encodes a membrane protein and acts cell-autonomously

INTRODUCTION

The first step in the development of the *Drosophila* nervous system is the specification of neural precursors (NP's) at precise locations. Groups of uncommitted ectodermal cells at particular positions acquire the potential to become NP's through expression of proneural bHLH transcription factors, which include the *achaete-scute* complex and *atonal* gene products. Within each of these groups of cells, called proneural clusters, a small subset of cells go on to express elevated levels of the proneural genes. Finally, proneural gene expression is restricted within each cluster to a single cell which adopts the neural fate; the rest of the proneural cluster cells stop expressing the proneural genes and adopt the epidermal fate. Loss of proneural gene function results in decreased numbers of NP's, while gain of function results in increased numbers of NP's (reviewed in Ghysen, 1993).

Experiments in the grasshopper have shown that cell-cell interactions are required for specifying only one NP within each cluster (Doe and Goodman, 1985). When the NP is ablated, another cell from the proneural cluster replaces it; the presence of the original NP prevents the other cells from becoming NP's, through a process called lateral inhibition. A variety of experiments in *Drosophila* support this lateral inhibition model (for review see Simpson, 1990). The concept of lateral inhibition has been extended to include the process in which cells of the proneural cluster compete with each other through cell-cell interactions, eventually selecting a single NP. Accordingly, each cell inhibits the ability of the other cells to become neural while increasing its own neural potential. This situation is unstable, and once one cell gains an advantage, it quickly adopts the neural fate, forcing all the other cells to become epidermal.

Mutations in a number of genes, known as the neurogenic genes, disrupt the lateral inhibition process, allowing many more cells to adopt the neural cell

fate than in the wild-type situation. The neurogenic genes are also required for the development of other tissues including somatic muscles, the oocyte, the wing, and the differentiated sense organ (reviewed in (Artavanis-Tsakonas et al., 1995). The neurogenic genes *Notch* (*N*), *Delta* (*Dl*), *Suppressor of Hairless* (*Su(H)*), and *Enhancer of split* (*E(spl)*), have been shown to interact genetically, and are thought to function in a common pathway (de la Concha et al., 1988; Vässin et al., 1985) . *mastermind* (*mam*) and *neuralized* (*neu*) mutations interact with some alleles of *N* and *E(spl)*, but it is unclear how they fit into the *N/Dl* pathway (de la Concha et al., 1988; Xu and Artavanis-Tsakonas, 1990)Knust *et al.*, 1987).

Many of the neurogenic genes encode proteins which have been implicated in signal transduction and cell fate specification. *N* and *Dl* encode transmembrane proteins with EGF-like repeats, and may act as receptor and ligand respectively (Kidd et al., 1986; Vässin et al., 1987; Wharton et al., 1985) *Su(H)* encodes a transcriptional regulator that interacts with the CDC10/ankryin repeats of N, and is thought to regulate *E(spl)* expression in response to N signaling (Bailey and Posakony, 1995; Fortini and Artavanis-Tsakonas, 1994; Lecourtois and Schweisguth, 1995) The *E(spl)* complex encodes seven bHLH transcriptional regulators and a nuclear protein with a WD-40 motif (Hartley et al., 1988), and *mam* encodes a nuclear protein which may also regulate gene expression (Smoller et al., 1990). *neu* contains a putative zinc finger and helix-turn-helix motifs, but it has no known biochemical function (Boulianne et al., 1991; Price et al., 1993).

As components of the lateral inhibition pathway, each of the neurogenic genes is involved in either generating the lateral inhibition signal or responding to it. This is most evident in the case of *N* and *Dl* which are thought to encode receptor and ligand respectively (Fehon et al., 1990; Heitzler and Simpson, 1991). The genes involved in responding to the signal (*i.e. N*) act cell autonomously,

while the genes involved in generating the signal (*i.e. Dl*) do not act cell autonomously.

The *big brain* (*bib*) gene is distinct from the other neurogenic genes. Most notably, *bib* has not been shown to interact with the other neurogenic genes (de la Concha et al., 1988). Further, loss of function *bib* mutations cause less severe neural hyperplasia than loss of function mutations in the other neurogenic genes (Lehmann et al., 1983) and do not cause defects in sense organ differentiation. The N-terminal half of the predicted *bib* gene product shares sequence similarity with a group of channel proteins which have not been implicated in either cell fate specification nor in cell-cell communication, while the C-terminal half does not share homology with any known proteins.

To further elucidate the function of *bib*, we have analyzed the *bib* transcript for function during neurogenesis, examined tissue-specific expression and subcellular localization of bib protein, and determined where *bib* activity is required during neurogenesis. We demonstrate directly that the *bib* cDNA encodes a functional protein that can the *bib* loss of function phenotype. The bib protein is membrane associated and expressed in tissues that require neurogenic gene activity for their development. Finally, we demonstrate that *bib* acts cell autonomously, functioning in epidermal cells to help maintain the epidermal fate and inhibit the neural fate. Combined with our observation that bib protein colocalizes with Dl during neurogenesis, these results suggest that *bib* is intimately involved in the reception of or response to the lateral inhibition signal.

MATERIALS AND METHODS

Drosophila stocks

All flies were raised at room temperature on cornmeal-agar medium unless otherwise noted. We used the following stocks: *hairy*-GAL4 (Brand and Perrimon, 1993), *bib*^{FX1}, *bib*^{C7a}, *bib*^{IDO5} and *yw*; Oregon-R (Lindsley and Zimm, 1992), and UAS-activated *N* (E. Giniger, unpublished). GAL4$^{109;68}$ was isolated in an enhancer trap screen (L. Luo and S. Ralls) and is expressed in sense organ precursors and their progeny (G. Feger and S. Ralls).

UAS-bib constructs

The c13 *bib* cDNA (Rao et al., 1990), as well as other independently isolated cDNAs were subcloned into the XhoI and XbaI sites of the pUAS vector (Brand and Perrimon, 1993). These constructs were transformed into *yw*; Oregon-R flies using standard methods.

Immunofluorescence, confocal microscopy and immunoelectron microscopy

We used the following antibodies: guinea pig anti-myc (G. Feger and Y.N. Jan, unpublished), guinea pig anti-ase (A. Jarman), monoclonal antibody 22c10 (Zipursky et al., 1984) Rabbit anti-bib was raised against a peptide containing the C-terminal predicted amino acids (672-701) of *bib* (QQQQQQQQQQMMMQQQQQHYGMLPLRPN). Antibody staining was absent from *bib* ^{C7a} and *bib* ^{FX1} mutant embryos, but still present in the developing mesoderm of *bib*^{IDO5} mutant embryos (data not shown). Mouse (Kooh et al., 1993) and guinea pig anti-Dl were kindly provided by the Muskovitch lab.

We performed immunofluoresence labelings according to (Rhyu et al., 1994) with the following modifications. We fixed all samples in PEMS (0.1M PIPES pH 6.9, 1mM EGTA, and 2mM $MgSO_4$) + 4% formaldehyde. Embryos were fixed for 20-30 min., while imaginal discs were fixed for 10-20 min. All samples were blocked in PBT + 2% normal goat serum. For some of our stainings, we amplified the signal using biotinylated secondary antibodies (Jackson Laboratories, U.S.A.), and streptavidin conjugated to DTAF, LRSC, or Cy-3. We mounted samples in glycerol/PBS/2% n-propylgallate, and examined them using a Bio Rad MRC-600 confocal microscope. Figures were prepared using Adobe Photoshop and Adobe Illustrator.

We performed immunoelectron microscopy as described in (Jongens et al., 1994)., using the anti-bib antibody.

Clearing embryos for cuticle analysis was performed as described in (Ashburner, 1989).

Clonal Analysis

We used the FLP/FRT method developed by (Golic and Lindquist, 1989) to generate mosaic clones. We constructed chromosomes with a y^+ P-element at 25, ck^{13} (Lindsley and Zimm, 1992), either bib^{FX1} or bib^{C7a} and p[ry^+ FRT] at 40A (Xu and Rubin, 1993). To generate the mitotic clones we mated *yw*: y^+ *ck bib* FRT/CyO males to hsFlp1; p[w^+ *myc*] FRT females (Xu and Rubin, 1993). Flp activity was induced in the progeny at various developmental stages by two shifts to 39°C for 30 minutes separated by 30 minutes at room temperature. Clones were examined in Flp1/*yw*; y^+ *ck bib* FRT/p[w^+ *myc*] FRT females. Control flies missing Flp1 or either FRT chromosome did not display clones.

RESULTS

(Rao et al., 1990) reported that they had isolated the *bib* transcript based on their findings that *bib* mutant chromosomes contained mutations in and around the proposed *bib* transcript, and that a 14kb genomic DNA fragment could rescue the lethality caused by *bib* mutations. To demonstrate unambiguously that the previously cloned *bib* cDNA encodes *bib* gene activity, we attempted to rescue the *bib* embryonic neurogenic phenotype by expressing the *bib* cDNA using the GAL4 system developed by Brand and Perrimon, (1993). This system allowed us to express *bib* in a variety of tissues by using previously established lines which express GAL4 in particular sets of cells.

Embryos mutant for *bib* develop a hypertrophic nervous system resulting in extreme reduction in the ventral cuticle (Lehmann et al., 1983); these embryos also have defects in a variety of other tissues (Corbin *et al.*, 1991; Hartenstein *et al.*, 1992 and data not shown). Using the *hairy*-GAL4 (*h*-G4) enhancer trap line IJ3 (Brand and Perrimon, 1993), we were able to express UAS-*bib* (Figure 2.1A). As described below, this expression was sufficient to rescue the *bib* neurogenic phenotype in a segmental manner. In fact, the rescued embryos were easily identified by their defective head cuticle, but relatively intact thoracic and abdominal cuticle. Segmental rescue allowed us to easily distinguish rescued embryos from wild-type and *bib* mutant embryos without requiring independent makers.

We generated bib^- embryos which express UAS-*bib* under *h*-G4 control using the scheme outlined in Figure 2.1B. 3/4 of the embryos (all those that were $bib^-/+$ or +/+, regardless of the assortment of *h*G4 and UAS-*bib*) were indistinguishable from wild-type (data not shown), and thus they hatched and crawled away. Of the unhatched embryos, 3/4 displayed the bib^- phenotype, while 1/4 developed significant ventral cuticle (Figure 2.1C). Most rescued

cuticles looked like those of pair-rule mutants, reflecting rescue of the ventral denticle belts in every other segment. A number of rescued embryos developed nearly wild-type thoracic and abdominal cuticle without any missing denticle belts (Figure 2.2B). We believe that this was due to UAS-*bib* expression below our level of detection throughout the embryo , and not due to non-autonomous *bib* function. This conclusion is supported by our observation that UAS-activated N, which acts autonomously, caused antineurogenic effects in all segments when driven by *h*G4. Head development of both rescued and neurogenic embryos was disrupted (compare Figure 2.1A to B and C), as expected from the absence of *h* expression from the procephalic neurogenic region. Control crosses in which we left out either UAS-*bib* or *h*-G4 did not yield any rescued embryos (Figure 2.1C and data not shown). Finally, rescue was not dependent on a particular UAS-*bib* insertion site, as we were able to demonstrate rescue using several independent UAS-*bib* lines. On the basis of these experiments, we conclude that UAS-*bib* expression can rescue the thoracic and abdominal cuticle defects in *bib* mutant embryos.

bib expression can rescue the CNS and PNS defects in *bib* mutant embryos

In addition to rescue of the cuticle phenotype, we observed rescue of the nervous system phenotype in *bib* mutant embryos which express UAS-*bib* driven by *h*G4. We collected embryos generated using the scheme in Figure 1B, and stained them with monoclonal antibody 22c10 which labels neurons (Zipursky et al., 1984); 303/450 (67%) of the embryos were wild-type, 114/450 (25%) showed the extreme *bib* neurogenic phenotype, and 33/450 (7%) of the embryos displayed a significantly milder neurogenic phenotype in the thorax and abdomen (Figure 2.1C and Figure 2.2D-F). The CNS hypertrophy in rescued

embryos was strikingly reduced when compared to the CNS hypertrophy in *bib*$^-$ embryos (data not shown).

As expected, rescue of the peripheral nervous system (PNS) showed parasegmental periodicity. Wild-type embryos almost invariably have 5 lateral chordotonal organs per hemisegment, while *bib*$^-$ embryos have an average of at least 10 per hemisegment. We observed nearly wild-type numbers of lateral chordotonal organs in T2, A1, A3, A5, and A7 (mean #= 5.1±0.6), but there were extra chordotonals in T1, T3, A2, A4, A6, and A8 (mean #= 8.0±1.5). The lateral chordotonal organs arise from the posterior compartment (Hartenstein, 1987), thus the segmental rescue of chordotonal hypertrophy is consistent with the *h* expression pattern in odd-numbered parasegments. Hypertrophy of the other sense organs in the PNS is decreased in the rescued embryos (Figure 2.2G-H).

UAS-*bib* does not rescue the other neurogenic mutants

We used schemes similar to that denoted in Figure 2.1B to assess the ability of UAS-*bib* to rescue the neurogenic phenotype in embryos mutant for other neurogenic genes. UAS-*bib* expression driven by *h*-G4 did not rescue the cuticle defects *Dl, N, E(spl), mam,* nor *neu* embryos (data not shown). In addition, UAS-*Dl* and UAS-*neu* did not rescue the neurogenic phenotype in *bib* mutant embryos. In contrast, UAS-activated *N* still had an antineurogenic effect in *bib* mutant embryos, consistent with the results of Lieber *et al.* (1993) using heatshock activated *N* . These results indicate that UAS-*bib* specifically rescues the defects in *bib* mutant embryos, providing conclusive evidence that the previously cloned *bib* cDNA does encode *bib* activity (Rao et al., 1990), and in addition, *bib* functions upstream of or parallel to *N*.

Subcellular localization of big brain protein

To determine whether the subcellular distribution of the *bib* gene product was consistent with its predicted structural similarity to integral membrane channel proteins, we raised a polyclonal rabbit antibody to a peptide encompassing amino acids 672-701 of the translated *bib* cDNA sequence (see Materials and Methods). Staining with this antiserum was specific for bib protein, as the signal was absent from embryos homozygous for the *bib* alleles FX1, C7a, and III9-5. Embryos mutant for one strongly neurogenic *bib* allele, ID05, still expressed protein in the mesoderm. Further, the bib antibody staining was also competed away by the *bib* peptide, but not by non-*bib* peptides (data not shown).

We observed bib protein in all plasma membranes just before cellularization of the blastoderm embryo. The pole cell membranes contained a small amount of bib which disappeared before gastrulation (not shown). After cellularization, bib was present in the basolateral membranes of all cells and concentrated in a ring around each cell demarcating the border between the apical and basolateral membranes where the cells are tightly apposed (Figure 2.3A). In tangential optical sections, these rings of staining produced a honeycomb pattern, and bib was essentially absent from the apical membrane (Figure 2.3A-B). In addition to the membrane staining, there was also punctate signal within the cytoplasm that may reflect bib protein within intracellular vesicles. Just before gastrulation, a ventral stripe of cells, corresponding to the prospective mesoderm, showed an increase in punctate signal and a decrease in plasma membrane signal (Figure 2.3C).

To examine whether bib was associated with specific structures within cells, we performed immunoelectron microscopy on embryos labeled with bib antibody and a secondary antibody conjugated to 1.4 nm gold particles (see

Materials and Methods). Using several fixation conditions, we consistently observed grains associated with the plasma membrane of all cells in the neurogenic ectoderm and clusters of grains within the cytoplasm, but very few grains within the nuclei (Figure 2.3D-E). The grains associated with the plasma membrane were predominantly on the cytoplasmic face, consistent with the predicted cytoplasmic location of the C-terminal antibody epitope (Rao et al., 1990). The clusters of grains in the cytoplasm were often associated with small vesicles (not shown) these clusters may correspond to the punctate signal observed in confocal micrographs (compare Figure 2.3D-E to Figure 2.3A-B). We also observed large numbers of grains associated with the apical adherens junctions described by Tepass *et al.* (1994). Figure 2.3D-E shows one such junction between two neuroectoderm cells in a stage 9 embryo.

A number of proteins involved in signaling are internalized and transported to multivesicular bodies. In *Drosophila*, these proteins include the receptor tyrosine kinase product of the *sevenless* gene, its ligand *bride of sevenless*, and the secreted product of the *wingless* gene (Cagan et al., 1992; González et al., 1991; van der Heuvel et al., 1989). With fixation conditions that gave strong plasma membrane signal, we did not find grains associated with multivesicular bodies in any of the cells of the neuroectoderm (not shown). The absence of signal probably indicates the absence of bib protein, although we cannot rule out inadequate penetration of the antibody, masking of the peptide epitope, or degradation of the protein in multivesicular bodies.

bib and Delta proteins colocalize

In the prospective mesoderm just before gastrulation, bib protein disappeared from the plasma membrane (Figure 2.3C), and was found in punctate cytoplasmic structures basal to the nucleus. The Delta protein is

distributed in a similar manner (Kooh et al., 1993). To address whether bib and Dl colocalize, we simultaneously labeled embryos with bib and Dl antibodies. The bib and Dl proteins did in fact colocalize in the punctate cytoplasmic structures of prospective mesoderm cells (Figure 2.4A-C), although the intensity of the two signals was not always similar.

After gastrulation, bib protein continued to be expressed in all cells of the neurogenic ectoderm. In addition to the membrane staining, we found striking punctate localization in the cytoplasm, which was once again coincident with Delta (Figure 2.4D-F). This localized staining could represent down-regulation of bib and Delta from the cell surface. In other tissues, and in cell culture, Dl is colocalized in punctate structures with N, even in cells that do not express Dl. Perhaps Dl undergoes receptor-mediated endocytosis (Kooh et al., 1993). We only observed punctate bib staining in bib-expressing cells, so it is unlikely that bib in one cell is endocytosed into adjacent cells.

To determine whether the punctate bib localization was functionally significant for lateral inhibition, we examined bib and Dl expression in *bib, Dl* and *N* mutant embryos. We found that punctate bib expression in the prospective mesoderm and neurogenic ectoderm was still present in *Dl* and *N* mutant embryos. In addition, punctate Dl expression was present in *bib* and *N* mutant embryos (data not shown). These results indicate that while the bib and Dl proteins colocalize to punctate cytoplasmic structures, this colocalization is not correlated with functional lateral inhibition.

bib expression during neuroblast segregation

To determine where and when bib might act during neurogenesis, we examined bib protein expression during neuroblast (Nb) segregation. bib was localized all along the basolateral cell membranes of neuroectoderm cells, and in

addition to being concentrated apically in the region of the adherens junctions, it appeared to be concentrated basally, where the cells contacted each other and the nascent Nb (Figure 2.5A). The basal concentration of bib protein may be important for efficient signaling between proneural cells.

By stage 9, bib protein was expressed at a much lower level in the developing CNS, but it continued to be expressed in the epidermis and was expressed in the cells of the mesoderm (Figure 2.5D-F), generating a "two-stripe" pattern similar to that described for Dl expression (Figure 2.5D-F and Kooh *et al.*, 1993). In stage 9 *N* mutant embryos where all the neuroectoderm cells have become Nb's, bib protein was only present in the mesoderm , confirming that Nb's lose bib expression. Confocal microscopy gave little indication of exactly when bib protein disappeared from the Nb's, because nascent Nb's are surrounded by the bib-containing membranes of adjacent epidermal cells (Figure 2.5G); however, in electron micrographs, there were far fewer grains associated with the membranes of segregating Nb's than with the membranes of adjacent neuroectoderm cells, indicating that bib was lost from the Nb membrane early during segregation (not shown). These results suggest that bib protein is not required in Nb's for maintaining the epidermal fate of the surrounding neuroectoderm cells.

bib is expressed in tissues that require neurogenic gene function

bib expression was maintained in the epidermis and mesoderm until stage 12. Consistent with this expression pattern, *bib* mutants embryos display severe defects in somatic muscles, peripheral glia, oenocytes, optic lobes, stomatogastric nervous system, salivary glands, Malpighian tubules, and dorsal vessel (Bate et al., 1993; Corbin et al., 1991; Hartenstein et al., 1992). We observed bib protein expression in all of these tissues or their precursors (Figure 2.6 and data not

shown). We also observed bib expression in the anterior and posterior midgut invaginations, ventral midline cells and tracheal pit cells (Figure 2.6A-B and not shown). While the midgut, ventral midline cells, and trachea are not defective in *bib* mutant embryos, they are all disrupted by loss of neurogenic gene function (Hartenstein et al., 1992; Menne and Klambt, 1994). Complementary expression of bib and Dl in rings of cells where the adult midgut precursors form in the proventriculus and at the midgut/hindgut boundary is strongly suggestive that these genes play a role in formation of the adult midgut. Although there was very strong bib antibody staining in a subset of hemocytes cells surrounding the esophagus (Figure 2.6C), it was probably due to a crossreacting antigen. Not only was the signal present in *bib*$^-$ embryos, but it was not competed away by *bib* peptide, and we observed no *bib* RNA expression in these cells (data not shown). Finally, we observed conspicuous bib expression in the adult muscle precursors and in subsets of the larval ventral nervous system and brain lobes; however, the significance of this expression is unclear.

In third instar wing discs, we found bib expression in the proneural clusters for the wing margin bristles, the sense organs along the dorsal radius and the sense organs of the notum (Figure 2.6D-F or G-I) where there is a known requirement for bib (Rao *et al.*, 1992 and this paper). bib was also expressed in 3-6 rows of cells at the dorsal ventral boundary of the wing pouch and in the cells that form the wing veins in late third instar wing discs. Despite the fact that *N* and *Dl* are required for wing margin and wing vein formation, loss of *bib* function does not disrupt these tissues (data not shown). Finally, bib was expressed at a high level in the adepithelial cells which form the flight muscle.

In summary, we have found that the bib protein expression pattern is consistent with the RNA distribution described by Rao *et al.* (1990). bib is expressed in essentially all tissues where neurogenic gene activity is required.

While bib embryos do not display a phenotype in all these tissues, the expression pattern suggests that bib functions in concert with the other neurogenic genes, or is at least involved in many of the same processes.

bib functions cell-autonomously to inhibit neural development

To begin to understand how a gene functions in a signaling pathway, it is essential to determine whether the gene is involved in producing the signal or responding to the signal. Proneural cluster cells compete with each other to become NP's, so it is possible to determine whether a gene is required to produce or to respond to the lateral inhibition signal by juxtaposing wild-type and mutant cells. If *bib* were involved in producing the signal, mutant cells should be able to receive the lateral inhibition signal from the adjacent wild-type cells and they should develop as epidermal cells. If *bib* were involved in responding to the signal, the mutant cells should be unable to receive the lateral inhibition signal from the adjacent wild-type cells, and they should develop as supernumerary neural cells.

Using the FLP/FRT system (Golic and Lindquist, 1989; Xu and Rubin, 1993), we generated homozygous *bib* mutant patches in bib^{-}/bib^{+} adults. We marked the patches with the recessive cuticle marker *crinkled* (Lindsley and Zimm, 1992). The *crinkled* (*ck*) mutation acts cell-autonomously, marking all the cells that form the adult cuticle; *ck* macrochaete (Mc) and microchaete (mc) form shortened and thickened hairs, while *ck* epidermal cells form multiple instead of single epidermal hairs (Figure 2.7A-B). The *ck* phenotype made it possible to determine the genotype of each cell at the clone borders.

bib mutant clones showed moderately increased numbers of Mc and mc (Rao *et al.*, 1992; Figure 2.7A-B and Table 2.1). The bib^{-} Mc and mc had normal sockets innervated by single neurons, so the extra bristles were not generated by

cell fate transformations within the sense organ precursor (SOP) lineage (not shown). Extra bristles were not likely due to extra divisions of the SOP's, because Rao *et al.* (1992) showed that the extra sense organs in *bib* mutant embryos are not due to extra divisions. In striking contrast to *bib*$^-$ clones, clones mutant for *N* and *Dl* show a greater degree of bristle hypertrophy, and strong alleles of *N* yield naked cuticle, presumably because all the daughters of the SOP's are transformed into neurons (Heitzler and Simpson, 1991).

At the *bib*$^-$ clone borders, supernumerary *ck* bristles, both Mc and mc, were found adjacent to *ck*$^+$ cells (Figure 2.7C). Normally, these ectopic bristles would have developed as epidermal cells, but without *bib* activity they became neural instead. This indicates that *bib*$^-$ cells could not always be instructed to become epidermal by adjacent wild-type (*ck*$^+$) cells, thus *bib* is required to respond to the inhibitory signal.

To determine whether *bib*$^-$ cells influence the neural versus epidermal fate decision of their heterozygous neighbors, we counted the numbers of wild-type and *ck* mc at the clone borders (Table 2.1). A mc was scored as being on the clone border, when it was adjacent to both *ck* and *ck*$^+$ epidermal hairs. When *bib*$^-$/*bib*$^-$ cells and *bib*$^-$/+ cells developed at clone borders, *bib*$^-$/*bib*$^-$cells produced sense organs three times as frequently as *bib*$^-$/+ cells, *i.e.* there were three times as many *ck* mc as *ck*$^+$ mc at the clone borders. The predominance of *bib*$^-$ mc at the clone borders was more than could be accounted for by the very mildly increased mc density within the clones (Table 2.1). Furthermore, at the borders of control clones marked with *ck*, we observed *ck* and *ck*$^+$ mc with equal frequency. These results indicate that *bib*$^-$ cells have at least wild-type signaling capability, and may even have increased signaling capability.

To confirm the cell autonomous action of *bib*, we examined *bib*$^-$ clones in late third instar wing discs. Using a myc-marked FRT chromosome (Xu and

Rubin, 1993), we were able to identify *bib*⁻ clones by the absence of myc staining (Figure 2.7E). Supernumerary SOP's arose adjacent to the clone borders, confirming that formation of the ectopic *bib*⁻ SOP's was not inhibited by the adjacent wild-type SOP's (Figure 2.7F). Our mosaic results in the adult cuticle and third instar wing disc indicate that *bib* is required for the full reception of or response to an inhibitory signal, and that it is not required for production of the signal.

Ectopically expressed *bib* and *Dl* interact synergistically

To determine whether *bib* can interact with *Dl*, we ectopically expressed *bib* and *Dl* in the SOP and its progeny cells using the $GAL4^{109;68}$. Rhyu (1994) has reported that UAS-activated *N*, driven by $GAL4^{109;68}$, causes a multiple socket phenotype in adult macrochaete and microchaete. This is the opposite phenotype from that caused by loss of *N* function and indicates that activated N can force all four SOP progeny cells to adopt the socket cell fate. UAS-bib driven by $GAL4^{109;68}$ causes a much milder hair to socket transformation phenotype, affecting only one or two macrochaete per fly. This demonstrates that even though *bib* is neither expressed nor required in the SOP lineage, it can function in the differentiation of these cells. In contrast, *Dl* whose loss of function phenotype is similar to that of *N*, generates no bristle differentiation defects when expressed using $GAL4^{109;68}$. Coexpression of UAS-bib and UAS-Dl causes a bristle phenotype nearly as strong as that of activated N (Figure 2.8). This observation indicates that *bib* can play an instructive role in cell fate determination, and it is also the first indication that *bib* may function in the *N* pathway; however, we cannot eliminate the possibility that *bib* functions in a parallel pathway.

DISCUSSION

bib cDNAs rescue the *bib* phenotype

Rao *et al.* (1990) isolated the *bib* gene by rescuing genomic DNA adjacent to a P-element which causes loss of *bib* function. The *bib* cDNA sequence predicts a gene product with similarity to the MIP family of small molecule channel proteins. This channel family includes many proteins, from the CHIP28 water channel in humans to the glycerol facilitator protein in *E. coli* (reviewed in Chrispeels and Agre, 1994). Many of the proteins have been shown to allow transport of water and small molecules like glycerol across membranes, but they have not been implicated in cell-cell communication nor cell fate decisions. We have provided direct confirmation that the unusual *bib* gene product is involved in the neural versus epidermal cell fate choice. In addition, rescue using *h*-G4 driven *bib* expression provides an excellent assay for in vivo *bib* gene activity. This assay can be combined with site-specific mutagenesis to determine which domains of the bib protein are required for function.

bib encodes a membrane associated protein that colocalizes with the Notch and Delta proteins

In all cells where bib protein is expressed, it is associated with the plasma membrane and punctate cytoplasmic structures. The punctate staining may represent bib protein in the endoplasmic reticulum, the Golgi, or vesicles on their way to and/or from the plasma membrane. As with *Dl* antibody staining, there is a striking increase in punctate staining in the cells of the prospective mesoderm, concomitant with loss of membrane staining. Kooh *et al.* (1993) have proposed that the increased vesicular staining represents down-regulation of *Dl* protein from the surface of these cells. We believe that bib is down-regulated in a similar manner.

Although *bib* and the other neurogenic genes share many of the same loss of function phenotypes, *bib* has not been shown to interact genetically with these genes. Even so, bib, Dl and N display similar patterns of subcellular localization, therefore bib functions in physical proximity to these proteins and might even interact with Dl and N directly or via other proteins and perhaps undergo receptor-mediated endocytosis in the Dl/N complex. Interaction with N is suggested by a phage presentation assay where M13 phage which express bib are enriched from a library of phage by binding to Schneider cells that express the extracellular domain of N (C. Wesley and M. Young, personal communication). It will be interesting to test for physical association between bib, N, Dl and the other neurogenic gene products using the yeast two-hybrid system (Chien et al., 1991), interaction assays in cultured cells (Fortini and Artavanis-Tsakonas, 1994) and by more conventional biochemical methods.

bib protein is expressed in tissues affected by neurogenic mutations

We have shown that *bib* protein is expressed in a pattern consistent with the previously described pattern for the *bib* transcript (Rao et al., 1990). It is expressed in all the cells of the neurogenic ectoderm before Nb segregation, consistent with its proposed role in lateral inhibition. The fact that bib expression turns off in the Nb's prior to division suggests that *bib* activity may be required only in the epidermal cells. Alternatively, bib may be required in all the cells, but only during the early events of Nb selection before segregation.

The bib expression pattern is also suggestive evidence that bib functions in concert with the other neurogenic genes. bib is expressed during the development of virtually all tissues defective in neurogenic mutant embryos, even in tissues such as the trachea that are not defective in *bib* mutant embryos. The fact that *bib* does not appear to be required in all tissues where it is expressed

may indicate that some tissues have lost their requirement for *bib*, and that expression in these tissues is just an evolutionary relic. Alternatively, there may be *bib* related genes which function in some tissues where *bib* is expressed, masking a loss of function *bib* phenotype. An equally likely explanation is that we have been unable to discern the subtle phenotype that loss of *bib* function causes in these tissues.

The neurogenic pathway uses different components that are adapted for the specific needs of each tissue. For instance, *Serrate* is a *N* ligand that functions specifically in the development of the wing and other tissues, but not during neurogenesis (Speicher et al., 1994). *bib* could also be one of these tissue-specific components; bib is not expressed in the peripheral sense organ lineage, and *bib* is not required for SOP differentiation.

bib is required in epidermal precursors to respond to the lateral inhibition signal

Our mosaic studies in adults provide strong evidence that *bib* is required autonomously in the epidermal precursors. In bib^{-} clones, supernumerary bristles are formed at the clone borders, immediately adjacent to wild-type cells, suggesting that when a bib^{-} cell develops adjacent to wild-type cells in a proneural cluster, it cannot consistently respond to the wild-type inhibitory signal, and therefore is more likely to become a NP. Thus *bib* function is required for the proper reception of or response to the lateral inhibition signal.

While *N* and *bib* both act autonomously, there are distinct differences in their mosaic phenotypes. The increase in bristle density in *bib* mutant clones is significantly milder than in *N* mutant clones (Heitzler and Simpson, 1991; Rao et al., 1992). This is consistent with the milder *bib* neurogenic phenotype in the embryo (Lehmann et al., 1983). A second difference is that bristles fail to form in clones of cells mutant for null *N* alleles where all four sense organ cells are

presumably transformed into neurons (Hartenstein and Posakony, 1990). Clones of cells mutant for null *bib* alleles show no bristle differentiation defects, consistent with the absence of PNS differentiation defects in *bib* mutant embryos. Finally, Heitzler and Simpson (1991) have shown that cells with lower *N* activity prevent adjacent cells with higher *N* activity from becoming neural. Complete loss of *bib* activity only modestly increases the ability of cells to inhibit their neighbors. These data further confirm that *bib* cannot be a universally required downstream effector of the *N* pathway, despite the fact that it is required in the signal receiving cells.

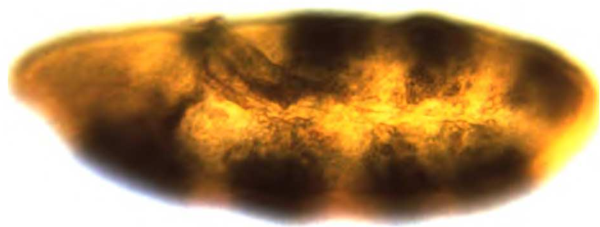
Models for *bib* function

We have provided evidence that *bib* is a membrane associated protein required for the accurate reception of or response to the lateral inhibition signal, and it is expressed in the right location to function in concert with the other neurogenic genes. We have also found that bib levels adequate to rescue the loss of function *bib* phenotype are not adequate to rescue the loss of function phenotypes of the other neurogenic genes. Further, *N* pathway activity cannot depend strictly on *bib* function, because the loss of function *bib* phenotype is only a subset of the loss of function *N* phenotype, and because the phenotype caused by expression of activated forms of N is not completely suppressed by loss of *bib* function (Lieber et al., 1993). These data constrain the mechanisms by which *bib* could act.

It has been proposed that *bib* functions in a pathway parallel to the *N* pathway , because bib does not interact with the other neurogenic genes (de la Concha et al., 1988). It is possible that *bib* is required for the reception of an epidermalizing signal that is produced not by the NP, but by the proneural cluster cells that will become epidermal precursors. *bib* could play an analogous

role in other tissues. The *bib* phenotype and protein distribution provide suggestive evidence that *bib* functions together with the other neurogenic genes. We favor a model where bib would act in the signal receiving cell to potentiate N/Dl binding or to potentiate the signal generated by N in response to Dl binding. Loss of *bib* function would cause a phenotype only when very efficient signaling was required; in other situations the phenotype would be negligible. This model predicts that genetic interactions between *bib* and *N* and/or *Dl* should be observed under the right conditions. Intriguingly, we have observed a synergistic interaction between *bib* and *Dl* expressed ectopically in the adult sense organ lineage. Using the functional cDNA, it will be possible to further elucidate the mechanism of bib action by examining the phenotypes of ectopic *bib* expression, defining the protein domains required for activity and screening for proteins that interact with bib.

A.



B. $\frac{bib^-}{CyO}$ X $\frac{h\text{-}GAL4}{TM3}$ $\qquad$ $\frac{bib^-}{CyO}$ X $\frac{UAS\text{-}bib}{TM3}$

$\frac{bib^-}{+}$; $\frac{h\text{-}GAL4}{+}$ X $\frac{bib^-}{+}$; $\frac{UAS\text{-}bib}{+}$

$\frac{bib^- \text{ or } +}{+}$; $\frac{h\text{-}GAL4 \text{ or } +}{UAS\text{-}bib \text{ or } +}$ $\qquad$ $\frac{bib^-}{bib^-}$; $\frac{+,\ h\text{-}GAL4 \text{ or } UAS\text{-}bib}{+}$ $\qquad$ $\frac{bib^-}{bib^-}$; $\frac{h\text{-}GAL4}{UAS\text{-}bib}$

C.

	bib alleles	UAS line	3/4 wild-type	3/16 neurogenic	1/16 rescued
22c10				% of all embryos	
	FX1/C7a		67% (303)	25% (114)	7% (33)
cuticle				% of dead embryos	
	FX1/C7a	1-1		83% (176)	17% (37)
	FX1/C7a	9-3		75% (159)	25% (54)
	C7a/C7a	1-1		86% (142)	14% (23)
	C7a/C7a	9-3		76% (155)	24% (50)
	C7a/C7a	-		97% (228)	3% (6)

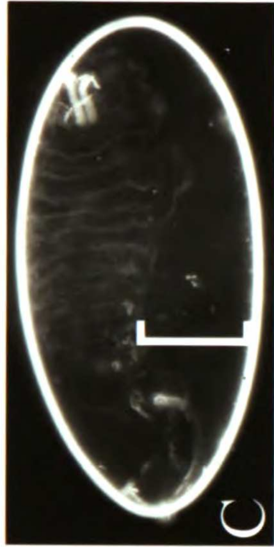
Figure 2.1 UAS-*bib* driven by *h*-G4 rescues the bib^{-} neurogenic phenotype.

(A) bib expression in a stage 9 bib^{C7a}/bib^{FX1}; *h*-G4 /UAS-*bib* embryo. bib protein is membrane associated and expressed in every other parasegment.

(B) Genetic scheme for rescue experiments.

(C) Results of rescue experiments using two independent UAS-*bib* insertions yielded the expected number of wild-type, neurogenic and rescued progeny, while those without UAS-*bib* yielded virtually no rescued progeny. Embryos were classified as rescued when they displayed the neurogenic head phenotype but also developed at least some ventral cuticle. The percentage of embryos with wild-type cuticles was not determined because the wild-type embryos hatched and crawled away.

In all figures, embryos are oriented with dorsal up and anterior to the left unless otherwise noted. Embryonic stages are according to Campos-Ortega and Hartenstein (1985).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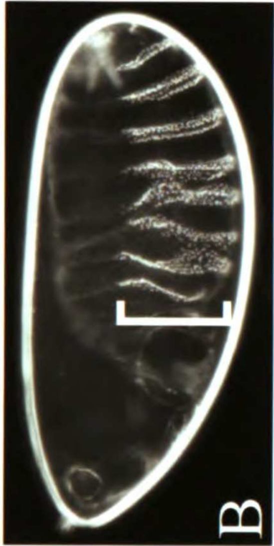
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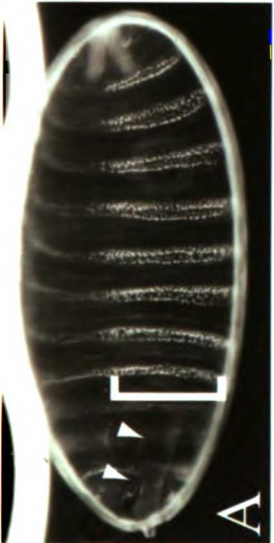
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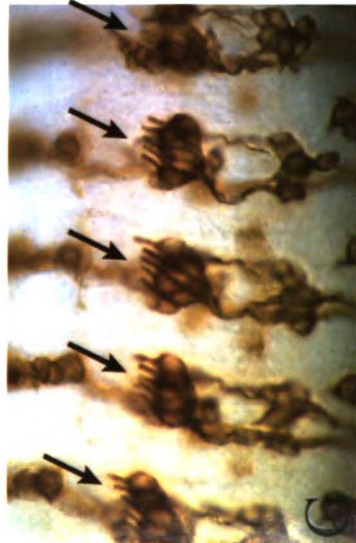
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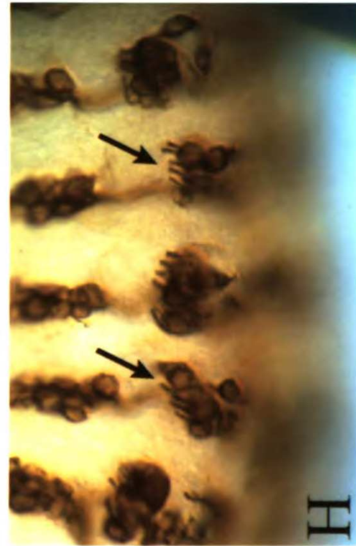

H

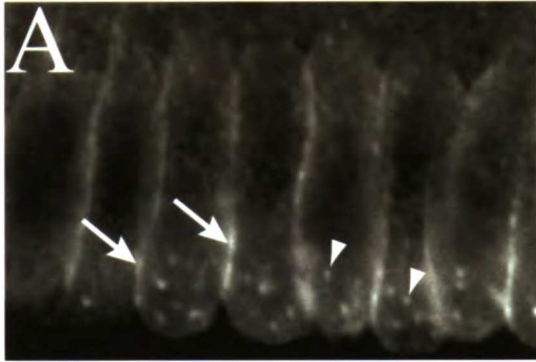
Figure 2.2 UAS-*bib* rescues the cuticle and nervous system phenotypes in *bib*⁻ embryos.

Wild-type (A, D and G), rescued (B, E and H) and bib^{C7a}/bib^{FX1} neurogenic (C and F) embryos generated using the scheme in Figure 1B.

(A-C) Rescued embryos develop ventral denticle belts (bracket in B), which are completely absent in their unrescued neurogenic siblings (bracket in C). Note that the head cuticle is defective and that the mouth hooks (arrowhead in A) fail to develop in both rescued and neurogenic embryos.

(D-F) Monoclonal antibody 22c10 staining for neurons reveals that rescued embryos (E) develop fewer neurons than their unrescued neurogenic siblings (F), but more than their wild-type siblings (D).

(G-H) The number of chordotonal neurons in alternate segments of rescued embryos (arrows in H) is nearly the same as in wild-type embryos (arrows in G).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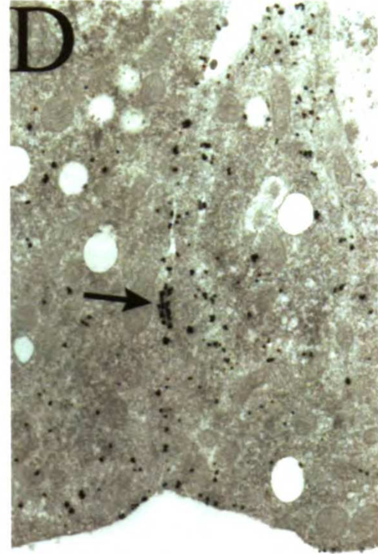
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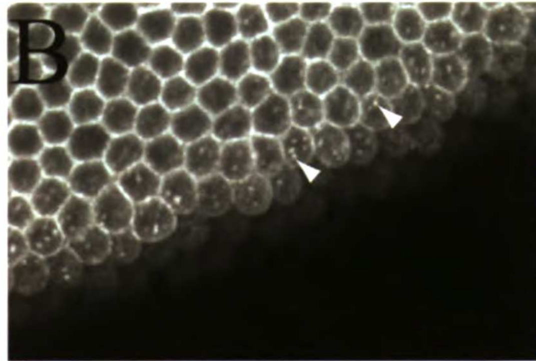
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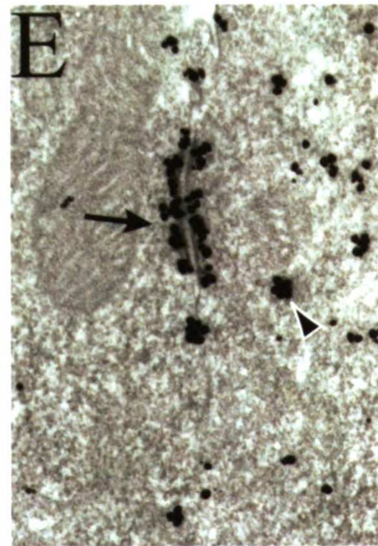
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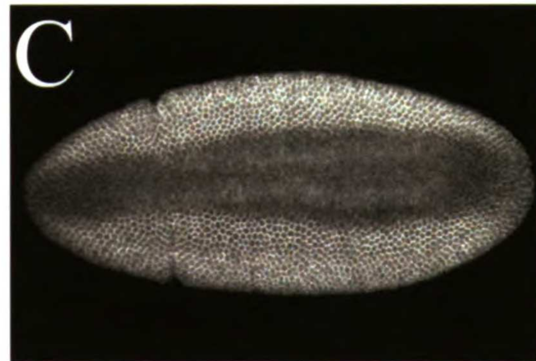
C

Figure 2.3 Subcellular localization of bib protein.

(A-C) Wild-type embryos stained with anti-bib antibody just before (A and B) and just after (C, ventral view) the start of gastrulation. In the cellular blastoderm, bib is present throughout the plasma membrane and concentrated at the border between the apical and basal membranes (arrows). bib is also present in punctate cytoplasmic structures (arrowheads in A and B). bib disappears from the membranes of prospective mesoderm cells at gastrulation (C).

(D-E) Immunoelectron micrographs of the neuroectoderm in a stage 8 embryo labeled with bib antibody (see Materials and Methods). Grains are typically found along the membranes, associated with adherens junctions (arrows) and in clusters within the cytoplasm (arrowheads).

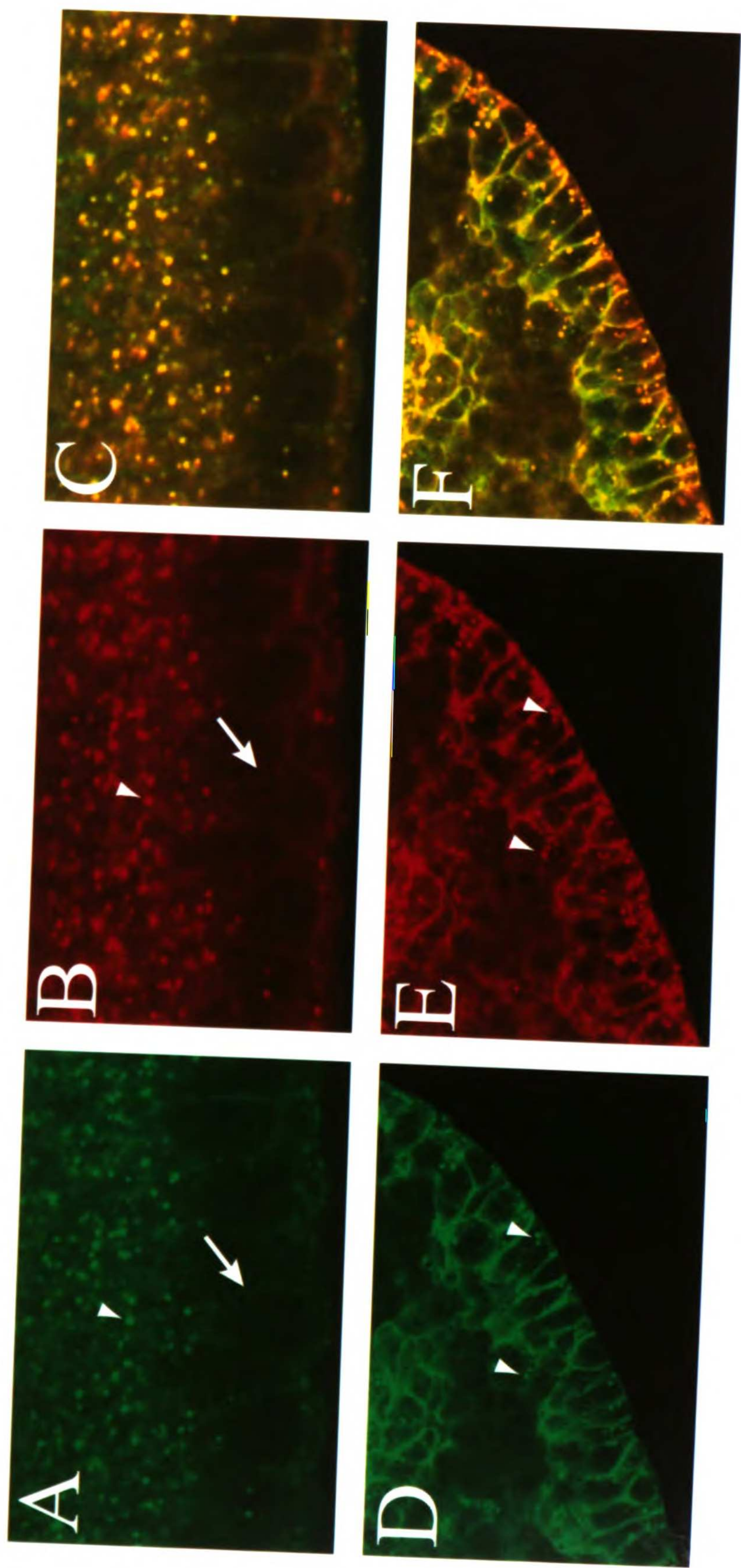
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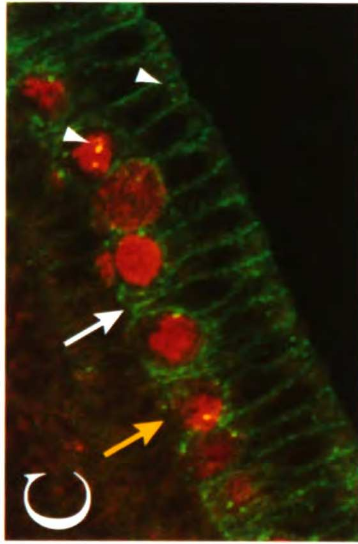
Figure 2.4 bib and Delta proteins colocalize in the prospective mesoderm and neuroectoderm cells.

(A-C) Prospective mesoderm cells in a wild-type embryo just before gastrulation. bib (green) and Delta (red) protein are absent from the plasma membranes (arrows) and concentrated at points within the cytoplasm of these cells (arrowheads).

(D-F) Neuroectoderm of a wild-type embryo during neuroblast segregation. bib and Delta proteins are present in the plasma membranes of neuroectoderm cells and neuroblasts, and are concentrated at points within the cytoplasm (arrowheads).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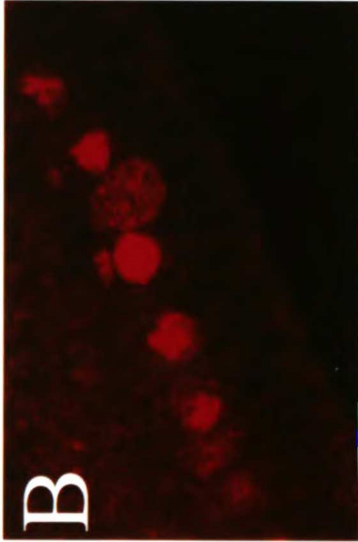
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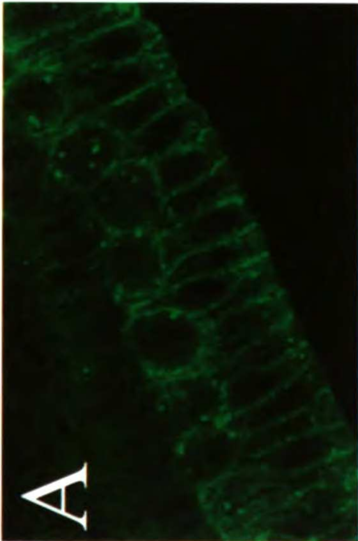
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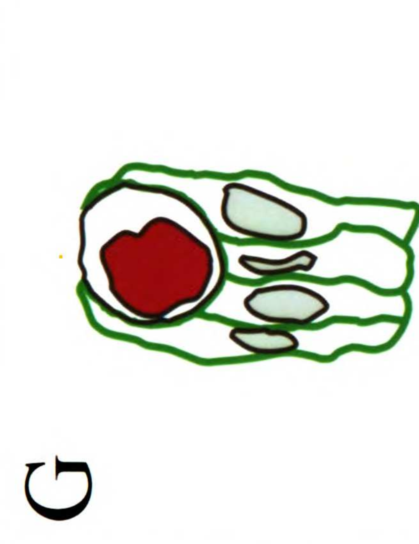
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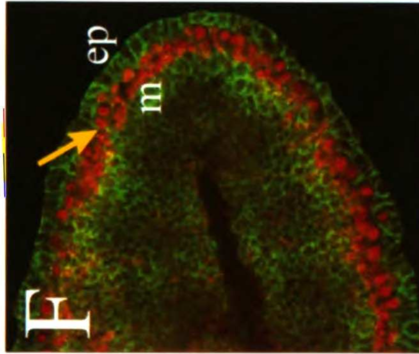
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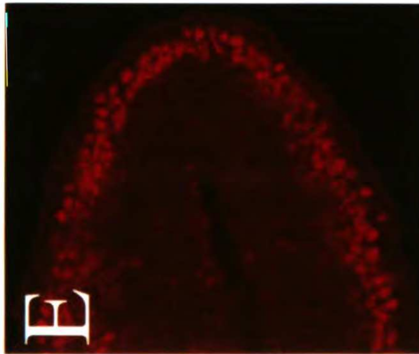
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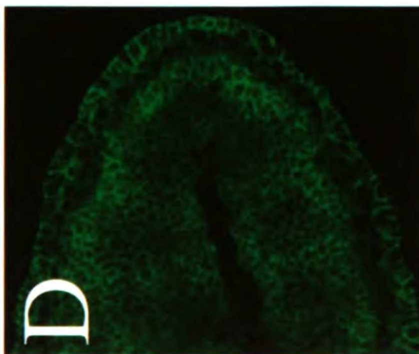
D

Figure 2.5 bib expression during neuroblast segregation.

Confocal micrographs of embryos labeled with anti-bib (green) and anti-ase (red) antibodies.

(A-C) bib and ase expression in a wild-type stage 9 embryo. ase is expressed in the nuclei of neural precursors and some of their progeny (Brand et al., 1993). bib is expressed throughout the plasma membranes of all neuroectoderm cells during neuroblast segregation and is concentrated basally where the cells contact the ase-expressing neuroblasts (white arrow). bib is also present in punctate structures in the cytoplasm of neuroblasts and neuroectoderm cells (arrowheads). Neuroblast membranes contain lower levels of bib than the surrounding neuroectoderm cells (yellow arrows in C and G).

(D-F) bib and ase expression in a wild-type stage 11 embryo. After neuroblast segregation, bib levels are lower in the developing CNS (yellow arrow) than in the overlying epidermis (ep) and the underlying mesoderm (m).

(G) Neuroectoderm cell membranes nearly surround the recently segregated neuroblast (traced from A).

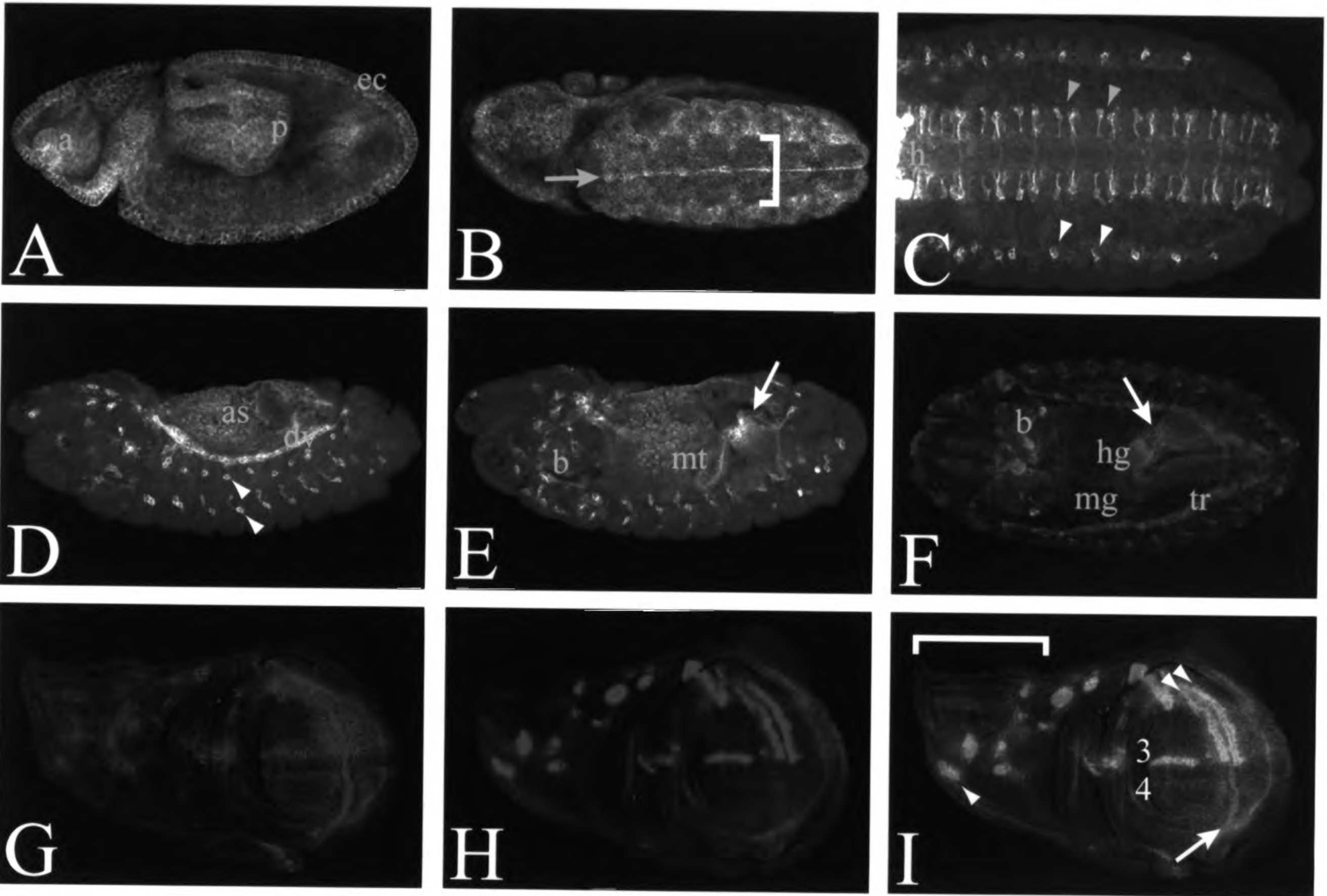

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Figure 2.6 bib protein is expressed in tissues that are defective in neurogenic mutant embryos.

(A) At stage 7, bib is expressed throughout the ectoderm (ec) and in the anterior (a) and posterior (p) midgut invaginations. Many cell types that derive from these regions are defective in neurogenic mutant embryos (Hartenstein et al., 1992).

(B) At stage 11 bib is still expressed at a high level throughout the epidermis and in the ventral midline cells (yellow arrow), but not in the developing CNS (bracket).

(C) Ventral view of a stage 16 embryo. bib is expressed in a segmentally repeated subset of ventral nervous system cells (yellow arrowheads) and in adult muscle precursors (white arrowheads). The strong signal in a subset of hemocytes (h) represents a crossreacting antigen (see text).

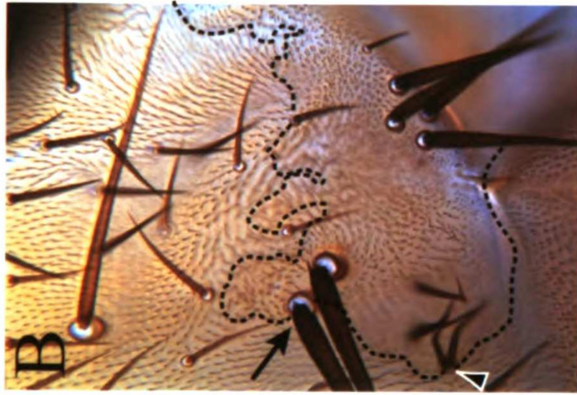
(D-E) Lateral views of a stage 13 embryo at two different focal planes. bib is expressed in the dorsal vessel (dv), Malpighian tubules (mt) and a subset of cells in the brain (b); these tissues are defective in bib mutant embryos (Hartenstein et al., 1992). bib is also expressed in the amnio serosa (as), adult muscle precursors (arrowheads) and posterior midgut (white arrow); phenotypes have not been described in these tissues.

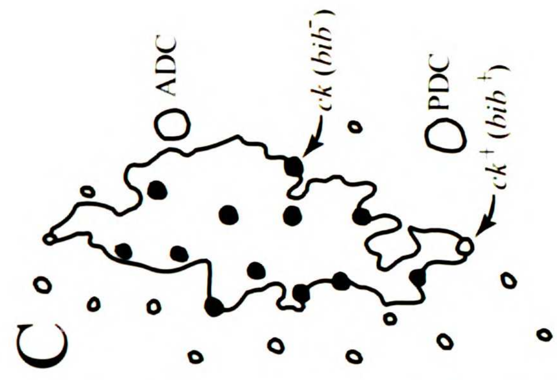
(F) Dorsal view of a stage 15 embryo. bib (green) is expressed at the posterior limit of the midgut (white arrow) adjacent to a domain of Dl (red) expression at the anterior limit of the hindgut (hg). bib is also expressed in a subset of cells in the brain (b) and at a low level throughout the midgut (mg). Dl is expressed at a high level in the trachea (tr).

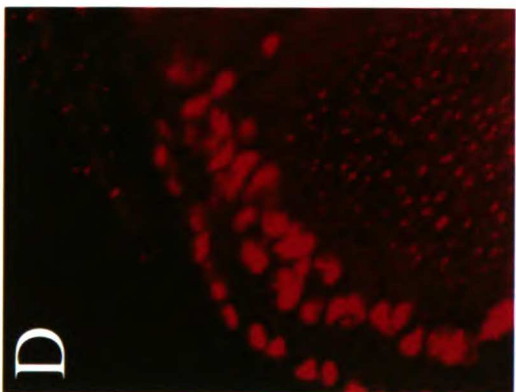
(G-I) Third instar ac-lacZ wing discs labeled with anti-bib (green) and anti-ßgalactosidase (red) which is expressed in proneural cluster cells. bib is expressed at a high level in proneural clusters (white arrowheads point to

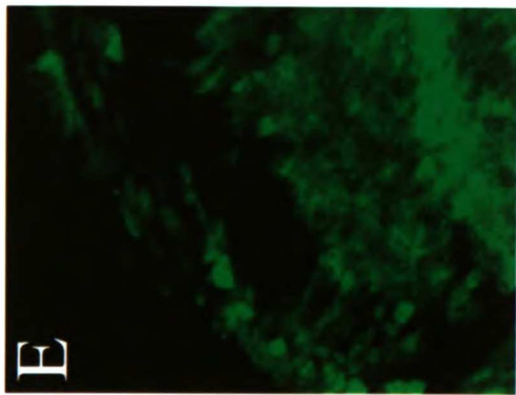
scutellar and wing margin clusters), cells at the dorsal/ventral compartment boundary (white arrow), prospective wing veins (labeled 3 and 4), and in adepithelial cells which become the adult flight muscles (bracket).

A


B


C
ADC
ck (bib$^-$)
PDC
ck$^+$ (bib$^+$)


D


E

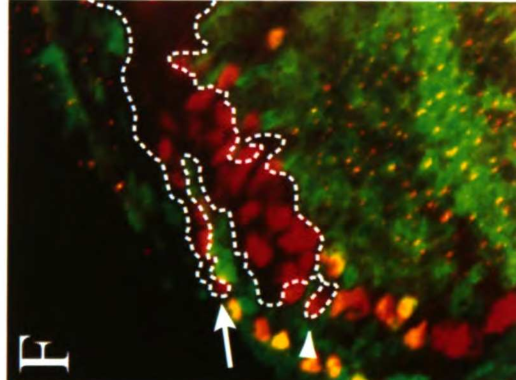

F

Figure 2.7 *bib* function is required cell autonomously.

(A-B) Homozygous bib^{FX1} clones marked with *ck* in the adult notum. Anterior is toward the top of the page; clone borders are marked with dotted lines.

(A) Supernumerary *ck* dorsocentral macrochaete (arrows) at the border between *ck* and ck^{+} epidermal hairs.

(B) Duplicated *ck* posterior dorsocentral macrochaete (arrow) at another clone border. Note also the duplicated *ck* microchaete with fused sockets at the clone border (arrowhead).

(C) Camera lucida drawing of a small bib^{FX1} clone. Homozygous *bib* mutant microchaete predominate at the clone borders (see Table 1).

(D-F) Homozygous bib^{FX1} clone encompassing part of the proneural region of the developing wing margin of a third instar wing disc labeled with anti-myc (green) and anti-ase (red). Anterior is toward the top of the page and dorsal is to the left. Cells in the clone do not express the myc marker, while wild-type twin spot cells express high levels of myc and $bib^{FX1}/+$ cells express intermediate levels. The clone (dotted line) includes a large segment of the dorsal proneural region and a row of precursors in the ventral proneural region (arrow). Note that supernumerary precursors form even when surrounded by myc-expressing *bib* cells (arrowheads). There is a second clone in the lower left corner of the image.

Table 2.1 Microchaete density is increased in *bib*$^-$ clones and *bib*$^-$ microchaete predominate at clone borders

FRT chromosome	epidermal hairs between microchaete within clones mean±S.D. (hairs counted)	*ck* microchaete adjacent to ck^+ hairs (microchaete counted)	ck^+ microchaete adjacent to *ck* hairs (microchaete counted)
bib^{FX1} ck	3.49±0.81 (118)	74% (95)	26% (34)
bib^{ID05} ck	3.66±1.01 (148)	72% (59)	28% (23)
bib^+ ck	4.64 ±0.98 (166)	52% (96)	48% (88)

SOP

wild-type	socket	hair	sheath	neuron
109-68; UAS-actN	socket	socket	socket	socket
109-68; UAS-Dl	socket	hair	?	?
109-68; UAS-bib	socket	hair	?	?
109-68; UAS-bib (rarely)	socket	socket	?	?
109-68; UAS-Dl and bib	socket	socket	socket	socket

Figure 2.8 The sense organ phenotype caused by *bib* and *Dl* coexpression mimics the phenotype of activated *N* expression.

The four cells in the adult sense organ derive from the sense organ precursor (SOP) through three asymmetric divisions which require the activity of ***numb*** and the *N* pathway (Hartenstein and Posakony, 1990; Rhyu *et al.*, 1994). Driving UAS-actN expression in the adult sense organ lineage with $GAL4^{109;68}$ transforms the non-socket cells into socket cells in nearly every adult external sensory (es) organ on the notum (Rhyu, 1994). Similar expression of UAS-Dl does not cause a multiple socket phenotype, and expression of UAS-bib causes a hair to socket transformation in only one or two es organs per fly; however, coexpression of Dl and bib causes a multiple socket phenotype comparable to that caused by actN expression in nearly every es organ. This is the first example of genetic interaction between *bib* and the *N* pathway.

CHAPTER 3

Delta is a ventral to dorsal signal complementary to Serrate, another Notch ligand, in *Drosophila* wing formation

INTRODUCTION

Cell-cell communication is a fundamental process required for patterning and growth during *Drosophila* wing development (for review see Whittle, 1990; Blair, 1995). The wing develops from a cluster of undifferentiated cells called the wing imaginal disc. The disc cells are set aside during embryogenesis, proliferate during larval development, and by late third larval instar, the disc has developed the basic pattern elements of the mature wing. Within the epithelial monolayer of the disc, dorsal and ventral compartment cells lie in two adjoining regions (Figure 3.2C). During pupal development, the dorsal cells become apposed to the ventral cells as the disc folds and everts along the dorsal/ventral (D/V) boundary to form the mature wing blade which is comprised of two symmetrical cell layers. The D/V boundary becomes the wing margin, marked with sensory bristles along the anterior margin and large non-innervated hairs along the posterior margin.

The wing consists of four compartments (anterior, posterior, dorsal and ventral) as defined by lineage restriction studies (Blair, 1993; Garcia-Bellido et al., 1973). Tissue excision and transplantation experiments have demonstrated that juxtaposition of cells from different regions of developing appendages induces proliferation and intercalation of pattern elements (for review see French et al., 1976). More recently, it has been proposed that interaction between cells with different compartmental identities is required for the normal growth and patterning of the wing and other appendages (Diaz-Benjumea and Cohen, 1993; Meinhardt, 1983; Tabata and Kornberg, 1994).

A number of genes involved in signaling between dorsal and ventral compartment cells have been identified. Dorsal but not ventral cells express *apterous* (*ap*), a homeodomain transcription factor which is required for wing disc proliferation and formation of the wing margin (Bourgouin et al., 1992; Diaz-

Benjumea and Cohen, 1993; Williams et al., 1993). The *fringe* (*fng*) gene, which encodes a novel, putatively secreted molecule, is expressed in the dorsal compartment under the control of *ap* (Irvine and Wieschaus, 1994). Juxtaposition of *fng* expressing and *fng* non-expressing cells induce tissue outgrowth and the formation of wing margin structures, suggesting that the fng signal can only be received by cells that do not express *fng*. To explain that both *fng*$^-$ and *fng*$^+$ cells at the borders of *fng*$^-$ clones are transformed into wing margin, Irvine and Wieschaus (1994) proposed that *fng* is a dorsal to ventral signaling molecule that induces a reciprocal (ventral to dorsal) signal from the *fng*$^-$ cells to the *fng*$^+$ cells. The existence of a reciprocal signal was also proposed by Williams et al. (1994) to explain the similar behavior of *ap* clones.

Candidates for the ventral to dorsal signal include *wg*, a member of the Wnt gene family, which encodes a secreted protein and is essential for wing development, as well as many other developmental processes in *Drosophila* (for review see Klingensmith and Nusse, 1994; Siegfried and Perrimon, 1994). Loss of function *wg* mutations result in abnormal wing phenotypes, ranging from loss of wing margin bristles to complete absence of wing tissue, depending on the particular combination of alleles (Phillips and Whittle, 1993 and reviewed in Lindsley and Zimm, 1992). In second instar wing discs, *wg* is expressed in the ventral compartment, and may function to maintain the restriction of *ap* expression to the dorsal compartment (Williams et al., 1993). Both *wg* and *ap* function are required for the expression of the *vestigial* (*vg*) enhancer lacZ reporter construct, the earliest molecular marker for the wing margin (Williams et al., 1994). Later, during third instar, *wg* expression is restricted to a stripe at the D/V boundary in cells which later form the wing margin. These results indicate that *wg* is required early for proliferation and/or patterning of the disc and later for formation of wing margin structures.

The *Notch* (*N*) signaling pathway is also essential for wing development. Loss of function mutations in *N* cause loss of wing tissue similar to that observed in *wg* mutants. *N* encodes a large transmembrane receptor necessary for cell-cell communication in a number of developmental processes. During neurogenesis, the *N* gene product, in concert with its ligand Delta (Dl), functions to single out neural precursors from fields of neuroectodermal cells (for review see Campos-Ortega, 1988; Ghysen et al., 1993; Artavanis-Tsakonas et al., 1995). This process requires cell-cell communication among groups of cells, all of which can both send and receive signals (Heitzler and Simpson, 1991). Strong genetic interactions between *N* and *wg* indicate that the two genes function in the same pathway during wing margin formation (Couso and Martinez Arias, 1994; Hing et al., 1994). Couso and Martinez Arias (1994) have proposed models in which *wg* acts upstream or parallel to *N* during wing margin development and may even be a N ligand; however, loss of *N* function on either side of the D/V boundary causes loss of wg expression, wing margin and wing blade tissue in both compartments (de Celis and Garcia-Bellido, 1994; Rulifson and Blair, 1995), suggesting a function for *N* upstream of *wg*.

Kim et al. (1995) and Diaz-Benjumea and Cohen (1995) have proposed that Serrate (Ser), a N ligand with sequence similarity to Dl (Fleming et al., 1990; Rebay et al., 1991), functions as a dorsal to ventral signal downstream of *fng*. *Ser* expression is restricted to the dorsal compartment in the second instar wing disc. Loss of *Ser* function in dorsal cells at the D/V boundary results in loss of wing margin, whereas ectopic expression of *Ser* in both the dorsal and ventral compartments induces adult wing tissue outgrowth and *wg* expression only in the ventral compartment (Speicher, et al., 1994; Kim et al., 1995).

The mild wing notching observed with temperature sensitive combinations of *Dl* alleles has implicated *Dl* in wing development, although its

role has remained unclear (Parody and Muskavitch, 1993). We demonstrate that *Dl* is required for wing development and can induce a number of genes required for wing formation including *wg, vg* and *cut*. We propose that *Dl* encodes a ventral to dorsal signal, because *Dl* is required in ventral cells at the D/V boundary and ectopic Dl induces *cut* expression and adult wing outgrowth only in the dorsal compartment. In contrast, ectopic Ser induces *cut* expression only in the ventral compartment, indicating that *N* expressing cells can have different responses to the two *N* ligands. These results suggest that *Dl* plays an equivalent but complementary role to *Ser* as a compartment-specific signal in the genetic program for wing margin development.

MATERIALS AND METHODS

Clonal Analysis

Adult mosaic clones of mutant *Dl* tissue were generated by X-irradiating second instar larvae, as described by Diaz-Benjumea and Cohen (1993). To mark the Dl^{+} chromosome, we used a $forked^{+}$ duplication on the third chromosome (distal to *Dl* at cytological map position 98B), kindly provided by F. Diaz-Benjumea and S. Cohen. This allowed us to identify *Dl forked* clones in flies hemizygous for f^{36a}. We used Dl^{rev10}, a loss of function *Dl* allele (Heitzler and Simpson, 1991), which gave no detectable Dl protein in homozygous Dl^{rev10} embryos.

For analysis in wing discs, mitotic clones lacking Dl were generated using flipase-mediated mitotic recombination (Golic, 1991; Golic and Lindquist, 1989). We recombined Dl^{rev10} and *ebony*, a recessive mutation which darkens cuticular structures, onto a chromosome carrying p[FRT, neo, ry^{+}] at 82B (Xu and Rubin, 1993). We crossed this chromosome into flies with a heat-shock myc marked p[FRT, neo, ry^{+}] third chromosome and a heat-shock flipase X chromosome, and generated homozygous clones in wing discs and adult wings (see Xu and Rubin, 1993). We identified *Dl* mutant clones by the absence of myc expression in wing disc cells. Many wing discs with clones induced during second instar were extremely distorted, making them difficult to analyze.

Immunocytochemistry

Larvae were dissected in PBS, fixed for 10 min in 4% formaldehyde, PEMS (0.1 M Pipes pH 6.9, 1mM EGTA, 2mM $MgSO_4$) and rinsed several times in PBT, blocked for 1h at room temperature with 2% normal goat serum and incubated over night at 4°C with the primary antibody. After several washes with PBT, fluorescent labeled secondary antibodies (Jackson Laboratories, USA) were

added for 1-2 hours at room temperature, washed with PBT, and mounted in glycerol/PBS/2% n-propylgallate. Samples were examined using a Bio Rad MRC-600 confocal microscope. Antibodies used: rabbit anti-ß galactosidase (Cappel, U.S.A.) mouse anti-Delta MAb 202 (Kooh et al., 1993), rabbit anti-vestigial (Williams et al., 1991), rat anti-cut (Blochlinger et al., 1988), rabbit anti-Notch (E. Giniger and Y.N. Jan, unpublished), rabbit anti-wingless (van den Heuvel et al., 1989), mouse anti-engrailed (Patel et al., 1989), rabbit anti-cubitus-interuptus (Schwartz et al., in press), guinea pig anti-myc (G. Feger and Y.N. Jan, unpublished). Confocal figures were assembled using Photoshop 3.0 (Adobe, USA) and Canvas 3.5.3 (Deneba, USA).

Ectopic Expression of *N* and *Dl*

Targeted ectopic expression of *N* and *Dl* was accomplished using the GAL4 system (Brand and Perrimon, 1993). UAS-*Dl* transgenic lines were generated by subcloning *Dl* cDNA 3.2 (Vaessin et al., 1987) into the pUAST vector and transformation into w^- flies by standard techniques. UAS-activated *N* lines were generated by subcloning the transmembrane and intracellular domain sequences of *N* fused to the *Dl* signal sequence into pUAST (E. Giniger, personal communication). To test for wild-type function of the UAS-*Dl* constructs we used hairy-GAL4 driven expression of UAS-Dl to rescue the neurogenic phenotype of Dl^{9P39} mutant embryos (D. Doherty and Y.N. Jan, in prep.). The UAS-activated *N* constructs displayed antineurogenic activity in embryos when expressed with hairy-GAL4, as expected for a constitutively active *N* construct (Lieber et al., 1993; Rebay et al., 1993; Struhl et al., 1993).

UAS-*Dl*, UAS-activated *N* and UAS-*N* (gift from L. Seugnet, M. Haenlin and P. Simpson) ectopic expression in imaginal discs was targeted using *ptc* GAL4 (Hinz et al., 1994) and other GAL4 enhancer trap lines. Homozygous

GAL4 flies were crossed to UAS-*Dl* /*TM6B, Tb* or homozygous UAS-*N* flies and grown at either 18°C, 22°C or 29°C, as indicated. Larvae carrying both the GAL4 and the UAS insertions were identified on the basis of the Tb^+ phenotype.

The expression pattern of ptcGAL4 was examined by crossing it to a UAS-*lacZ* reporter line carrying a nuclear localized beta-galactosidase under UAS control.

Genetics and Temperature Shifts

To test for N^{ts} suppression of the ptcG4-Dl adult phenotype, we crossed *w*, N^{ts}/*FM6*; *ptc* GAL4 females to *y w*; UAS-*Dl* /*TM6B, Tb* males in vials. We collected eggs in vials over a period of 24 hours (at 22°C), incubated the vials at 22°C until the larvae had developed to second instar, and then shifted the vials to 29°C for 24 hours. A range of abnormal wing outgrowth phenotypes was observed in both classes (*yw*/N^{ts} and *yw/FM6*) of ptc-Dl females, while none of the *w* N^{ts}/Y; ptc-Dl males had abnormal wing outgrowth.

All flies were grown on standard cornmeal-agar medium at room temperature unless otherwise noted. Mutations not specifically discussed here are described in Lindsley and Zimm (1992).

RESULTS

Loss of *Delta* function during wing development causes loss of wing tissue

As part of the *N* signaling pathway, *Dl* plays an important role in several developmental processes (for review see Muskavitch, 1994). Using a temperature sensitive allele of *Dl*, Parody and Muskavitch (1993) have shown that *Dl* is required during late second and early third instar for wing margin formation. Exposure of such *Dl* mutant flies to the restrictive temperature at this developmental stage leads to notching at the distal tip of the wing, similar to the phenotype seen in flies heterozygous for a *N* null mutation. To further analyze the role of *Dl* in wing development, we generated clones homozygous for a loss of function *Dl* allele, Dl^{rev10} (Heitzler and Simpson, 1991), by X-irradiating second instar larvae. We found that *Dl* is required for wing margin formation in the ventral but not the dorsal compartment. Ventral clones that abutted the D/V boundary caused gaps in the wing margin (Figure 3.1B, D and E and Table 3.1), whereas clones that abutted the boundary from the dorsal side and those within the wing blade in either compartment did not cause gaps in the wing margin. The loss of wing margin phenotype is nearly identical to that caused by clones lacking *Ser* (Figure 3.1B and Diaz-Benjumea and Cohen, 1995; Speicher et al., 1994). Clones were identified using *forked* as a marker (Diaz-Benjumea and Cohen, 1993) and by scoring hypertrophy of the wing veins caused by loss of *Dl* function (Figure 3.1C-D and Parody and Muskavitch, 1993). Loss of sensory bristles of the anterior wing margin and the non-innervated posterior wing margin hairs was caused by both dorsal and ventral clones that included the domains of cells that give rise to these cuticle structures. All clones, both dorsal and ventral, also caused hypertrophy of vein tissue when they overlapped the normal position of wing veins. The phenotypes in bristle differentiation and wing vein formation are consistent with the known functions for *Dl* (Parks and

Muskavitch, 1993; Parody and Muskavitch, 1993). The absence of large portions of the wing in *Dl* mosaics indicates that *Dl* is required for formation of the wing margin as well as the proliferation and/or viability of wing blade cells. The compartment-specific requirement for *Dl* is the first indication that *Dl* encodes a ventral to dorsal signal reciprocal to the dorsal to ventral *Ser* signal (Diaz-Benjumea and Cohen, 1995; Kim et al., 1995).

Loss of *Delta* function during wing development alters *wg* expression

Since our mosaic studies indicated that *Dl* plays an important role in wing development, we used *wg* expression as a marker to examine how loss of *Dl* function in mitotic clones affected formation of the D/V boundary. *wg* is expressed in the ventral compartment of second instar wing discs and then along the D/V boundary from early third instar onward (Baker, 1988; Couso et al., 1993; Williams et al., 1993). Adult viable *wg* alleles cause loss of the entire wing and occasional wing to notum transformations (reviewed in Lindsley and Zimm, 1992); however, reduced *wg* activity during 3rd instar causes loss of wing margin structures only (Phillips and Whittle, 1993).

We generated homozygous Dl^{rev10} clones in second instar wing discs and examined wg protein expression in these discs at late third instar using an anti-wg antibody (van den Heuvel et al., 1989). We identified clones by the absence of heat-shock induced expression of a nuclear myc marker on the Dl^{+} third chromosome (see Methods). *wg* expression was altered when mutant clones intersected the D/V boundary. In large clones, we consistently observed a reduction in *wg* expression at the D/V boundary in homozygous Dl^{rev10} cells that were surrounded by other homozygous Dl^{rev10} cells (not shown). At the borders of narrower clones, in the homozygous Dl^{rev10} cells that were adjacent to heterozygous Dl^{rev10} cells, D/V boundary *wg* expression was elevated and

ectopic *wg* expression extended into the ventral compartment (Figure 3.2A-B). The separation between wg and myc expression is due to the membrane association of wg and nuclear localization of myc. Rarely, low levels of ectopic *wg* expression extended for short distances into the dorsal compartment along the inside border of clones. These results suggest that *Dl*, like *fng* (Irvine and Wieschaus, 1994), can induce *wg* expression at the border between *Dl* expressing and *Dl* non-expressing cells.

Dl expression is elevated at the D/V boundary in second instar wing discs

The effect of *Dl*$^-$ clones on *wg* expression raises the question of whether *Dl* plays an early role in setting up the D/V boundary, so we examined Dl expression in wing discs during second and third instar when proliferation and wing margin formation occur. To characterize *Dl* expression with respect to the dorsal and ventral compartments, we stained discs expressing an ap-lacZ enhancer trap, which marks cells of the dorsal compartment (Diaz-Benjumea and Cohen, 1993), with antibodies to ß-galactosidase and Dl (Kooh et al., 1993). The earliest patterned Dl protein expression appeared during mid-second instar. The highest levels of Dl were centered along the D/V boundary, as marked by the limit of the ap-lacZ domain (Figure 3.2D-F). High levels of Dl protein were also present in the ventral compartment of the wing pouch, with lower levels in the dorsal compartment. Dl was absent from the region of the disc that forms the dorsal notum. In early 3rd instar wing discs, there was a small amount of punctate Dl staining in a narrow stripe of cells at the D/V boundary (not shown). Finally, at late 3rd instar, Dl expression was highest in two stripes flanking the *wg* expressing cells at the D/V boundary, as well as in the prospective wing veins and proneural clusters (Kooh et al., 1993 and Figure 3.2G-I). The *Dl* expression pattern is consistent with *Dl* having an early role in setting up the

wing margin and a later role in maintaining the wing margin and patterning the wing margin bristles.

Ectopic expression of *Delta* results in abnormal outgrowth of dorsal wing tissue

Having found that *Dl* is required for wing margin formation, we examined whether ectopic *Dl* expression could induce an ectopic wing margin or wing tissue outgrowth. Using the UAS/GAL4 system (Brand and Perrimon, 1993), we ectopically expressed a UAS-Dl transgene in the wing disc with the patched-GAL4 enhancer trap line G559.1 (ptcG4) (Hinz et al., 1994). We refer to the combination of ptcG4 with UAS-Dl as ptcG4-Dl. ptcG4 is expressed strongly in a stripe along the anterior/posterior (A/P) border of the wing disc by mid third instar, with the highest level at the sharp posterior border and gradually lower levels toward the more irregular anterior border (see Methods and Kim et al., 1995). The UAS/GAL4 system has been reported to give more extreme ectopic expression phenotypes at higher temperatures (Speicher et al., 1994). When raised at 29°C, all of our UAS-Dl lines were lethal in combination with ptcG4. At 22°C, one line (UAS-Dl^{30A1}) produced viable ptcG4-Dl adults with disrupted anterior cross veins. Shifting ptcG4-Dl^{30A1} larvae to 29°C for 24hrs during second instar resulted in adult flies with striking wing outgrowth. The abnormal wing tissue outgrowth occurred only on the dorsal side of the wing blade (Figure 3.3A-B); patches of large bristles characteristic of the anterior double row or posterior wing margin were present at the distal tip of each outgrowth. We found even more extreme outgrowth in the wings of ptcG4-Dl^{30A1} pharate adults raised at 29°C throughout development. Other UAS-Dl insertions crossed to ptcG4 yielded pharate adults with extreme wing outgrowth even when raised at 22°C. These pharate adults also displayed severe defects in

the legs, heads, nota and male genitalia. Thus, ectopic expression of *Dl* can induce wing outgrowth and a new wing margin, as well as defects in other tissues.

Ectopic *Delta* acts through *Notch*

Dl has been shown to signal through the N receptor during neuronal precursor selection, and this signaling is sensitive to levels of both N and Dl (Heitzler and Simpson, 1991; Vaessin et al., 1987). To determine whether ectopic *Dl* also utilizes the *N* signaling pathway for induction of wing tissue outgrowth, we asked whether outgrowth depended on wild-type *N* function. We used the N^{ts} mutation to reduce *N* activity; raising the temperature to 29°C for 24hrs during late second and early third instar larval development resulted in notching of the wing characteristic of the *N* mutant phenotype (Figure 3.3D and Shellenbarger and Mohler, 1978). Shifting second instar ptcG4-Dl male larvae that carried the N^{ts} mutation to 29°C for 24hrs completely suppressed the wing outgrowth phenotype, whereas control ptcG4-Dl flies that were N^{+} or heterozygous for N^{ts} displayed a strong outgrowth phenotype (Figure 3.3C).

Further evidence that ectopic Dl functions through N is that ectopic N expression also induces hairs characteristic of the wing margin, similar to the phenotype of ptcG4-Dl flies raised at 25°C. We ectopically expressed wild-type UAS-*N* (gift from P. Simpson) by crossing it to ptcGAL4 (ptcG4-N). ptcG4-N flies raised at 29°C throughout development had a short row of ectopic hairs along the fourth wing vein on both the dorsal and ventral sides of the wing blade, reminiscent of anterior double row or posterior wing margin hairs (Figure 3.3E-F); these flies occasionally displayed mild wing tissue outgrowth (data not shown). Taken together, these data confirm that ectopic Dl acts through N, its known receptor, to induce abnormal wing outgrowth.

Ectopic expression of *Delta* induces *wg, vg,* and *cut* expression

Our mosaic studies indicated that *Dl* may be able to induce *wg* expression at the border between *Dl* expressing and *Dl* non-expressing cells. To examine this possibility further, we looked for ectopic *wg* expression in third instar ptcG4-Dl wing discs. Indeed, *wg* was induced mainly along the posterior border of the ectopic *Dl* stripe, and rarely at lower levels along the anterior border. In the dorsal compartment, *wg* was induced in the cells just inside and just outside the posterior border of the ptcG4-Dl stripe, while in the ventral compartment, *wg* was induced at a lower level and only outside the stripe (Figure 3.4A-C). Induction of *wg* was not limited to lines which expressed GAL4 at the A/P boundary; lines driving UAS-Dl expression at other locations within the wing pouch also induced *wg* expression and disc tissue outgrowth, but not adult wing outgrowth (data not shown). We did not observe *wg* induction outside the wing pouch, suggesting that there are region specific factors that modulate the ability of *Dl* to induce *wg* expression.

In third instar wing discs, ectopic *Dl* expression also induced *cut, vestigial* (*vg*), *deadpan* (*dpn*) and *big brain* (*bib*) in cells along the ptcG4 stripe (Figure 3.4D and additional data not shown). While all four of these genes are normally expressed in the prospective wing margin (Bier et al., 1992; Blochlinger et al., 1993; Williams et al., 1991; D. Doherty in prep.), only *cut* and *vg* have wing phenotypes (reviewed in Lindsley and Zimm, 1992). There was a notable difference in the ectopic expression of *cut*, which was induced only in the dorsal compartment of the wing pouch (Figure 3.4E and Table 3.2), whereas *vg, bib* and *dpn*, like *wg*, were induced in both the dorsal and ventral compartments (Figure 3.4A-D and data not shown). Furthermore, these discs were distorted by tissue overgrowth in both the dorsal and ventral compartments (compare Figure 3.4A-C to Figure 3.2G-I); however, adult wing outgrowth was exclusively dorsal

(Figure 3.3A-B). Our observations indicate that many genes, including *wg* , *vg* and *cut*, can be downstream targets of *Dl* in the wing disc and that *Dl* has different effects in the dorsal and ventral compartments.

Since ptcG4-Dl wing discs displayed such striking outgrowth, we asked whether compartmental organization was disrupted. We examined expression of *cubitus interuptus*, a gene expressed in the anterior compartment, *engrailed*, a gene expressed in the posterior compartment, and ap-lacZ, an enhancer-trap expressed in the dorsal compartment, to determine whether ectopic Dl expression causes general reorganization of the disc. We found that despite the dramatic dorsal and ventral disc tissue outgrowth associated with ectopic *Dl* expression, both A/P and D/V compartmental organization appeared undisturbed (data not shown). Our results indicate that Dl is able to induce many of the aspects of the normal wing margin program including *wg*, *vg*, and *cut* expression, as well as wing tissue outgrowth and wing margin bristle formation.

Ectopic expression of *Notch* induces *wingless* expression

We examined ptcG4-N wing discs to determine whether ectopic *N* expression induced *wg* and *cut* in a manner consistent with its role as a receptor for Dl. Immunohistochemical labeling of ptcG4-N third instar wing discs with anti-N and anti-wg antibodies revealed induction of *wg* expression in a short stripe perpendicular to the D/V boundary that gives rise to the wild-type wing margin (white arrowheads in Figure 3.4F). ptcG4-N induced *wg* and *cut* in many fewer cells than ptcG4-Dl. *wg* and *cut* induction occurred in both dorsal and ventral cells, but only close to the prospective wing margin (white arrowheads in Figure 3.4F and H). Whereas it is formally possible that the levels of *N* activity were not sufficiently high to induce *wg* along the entire ptcG4-N stripe, a more

likely explanation is that the ectopically expressed N receptor was only activated in the cells that are in contact with cells near the wing margin which express Dl and Ser, known ligands for N (yellow arrowheads in Figure 3.2G). In other words, endogenous Dl and/or Ser may activate the ectopically expressed N in the ptcG4-N stripe, inducing *wg* and *cut* expression.

wingless is induced at borders between _Delta_ expressing and non-expressing cells

In ptcG4-Dl wing discs, *Dl* was ectopically expressed at a high level in a stripe several cells wide, and endogenous *N* is expressed throughout the disc; however, *wg* was not induced throughout the ptcG4-Dl stripe. In the ventral compartment, *wg* was induced mainly in the cells adjacent to the posterior border of the stripe (Figure 3.4A-C), indicating that *Dl* expressing cells do not receive the *Dl* signal. In the dorsal compartment, *wg* was induced in cells along the posterior border of the ptcG4-Dl stripe, in cells outside as well as within the stripe. One explanation for *wg* induction within the stripe is that *Dl* induces a reciprocal signal from the cells outside of the stripe (see Discussion). This reciprocal signal would be capable of inducing *wg* within the ptcG4-Dl stripe but not *cut*, as *cut* was expressed only in cells outside of the stripe (Figure 3.2E). Restriction of *wg* induction to the posterior edge of the ptcG4-Dl stripe cannot be due to factors specific to the A/P boundary, since we also observed *wg* induction at the borders between *Dl* expressing and non-expressing cells generated by GAL4 lines expressed at other locations.

One possible explanation for these observations is that Dl autonomously inhibits the ability of a cell to receive Dl signal from other cells. To determine whether increasing the level of N would allow Dl-expressing cells to receive Dl signal, we co-expressed N and Dl using ptcG4. Indeed, *cut* was expressed

throughout the width of the ptcG4 stripe in these discs (Figure 3.5A-C), indicating that N was activated by Dl throughout the stripe. *cut* expression was still restricted to the dorsal compartment, as in ptcG4-Dl discs, but it was no longer induced in cells outside the ptcG4 stripe. It is possible that N expressed within the stripe binds most of the Dl within the stripe, preventing signaling to the adjacent cells that express N at a lower level. These results provide evidence that Dl within a cell can inhibit Dl signal reception by that same cell, and that the ratio of Dl to N within a cell may determine its ability to both send and receive the Dl signal.

***Dl* and *Ser* have different signaling abilities in the dorsal and ventral compartments**

Dorsal and ventral cells respond differently to ectopic Dl expression (Table 3.2). Dl induces higher levels of *wg* in the dorsal compartment than in the ventral compartment, and *cut* expression and adult wing outgrowth are restricted to the dorsal compartment. Even when high levels of Dl and N are co-expressed in the ventral compartment, *cut* expression and adult wing outgrowth are not induced in the ventral compartment (Figure 3.5A-C and data not shown). Similarly, dorsal and ventral cells respond differently to ectopic Ser expression (Speicher et al., 1994; Kim et al. 1995 and Table 3.2). Ser-induced *wg*, *cut*, and *vg* expression, as well as both disc and adult wing outgrowth, are restricted to the ventral compartment (Kim et al., 1995, Table 3.2, and data not shown). Ser can partially substitute for Dl during neurogenesis in the embryo (Gu et al., 1995), and Dl and Ser have been shown to bind the same EGF repeat in N (Rebay et al., 1991), thus the Dl-induced *wg* and *vg* expression in the ventral compartment may indicate that Dl can partially substitute for Ser. Nonetheless, ectopically

expressed Dl and Ser have strikingly different effects in the dorsal and ventral compartments.

Is the specificity due to compartmental differences in receptor-ligand interactions or is it due to compartmental differences in the downstream response of the N pathway? To answer this question, we expressed a constitutively active truncated N protein (actN) using ptcG4. At 16°C, ptcG4-actN animals die as early pupae; however, third instar wing discs displayed extreme outgrowth in both dorsal and ventral compartments, and *cut* was induced equally in the dorsal and ventral compartments (Figure 3.5D-F). This result indicates that the factors responsible for compartment-specific N signaling act on or upstream of the N receptor.

DISCUSSION

We have shown that *Dl* is required during *Drosophila* wing development as a ventral to dorsal signal. Mitotic clones lacking *Dl* that include cells on the ventral side of the D/V boundary cause loss of wing margin and blade tissue in both compartments, and Dl protein is elevated at the D/V compartment boundary of second instar larval wing discs. Further, ectopic Dl expression induces wing margin formation and wing tissue outgrowth. In light of these findings we discuss the role of other genes in the N signaling pathway, the likely involvement of A/P boundary signaling components, and the requirement for a border between signaling and receiving cells at the D/V boundary during normal wing development. We also propose a model for *Dl* and *Ser* function during the early steps of wing margin formation in the second instar wing disc.

The *Dl-N* signaling pathway is required for wing formation

The *Dl-N* pathway consists of a cassette of genes which functions to transmit signals between cells at many stages during development (Artavanis-Tsakonas et al., 1995; Jan and Jan, 1993), and we have shown that Dl plays an essential role in wing development, probably by activating N . We propose that in addition to *N, Dl* and *Ser*, other genes in the cassette are likely to be involved in wing development. *Suppressor of Hairless* [*Su(H)*] is downstream of *N* in the signaling pathway, and Su(H) protein has been shown to translocate from the cytoplasm to the nucleus upon activation of N by Dl in transfected S2 cells (Fortini and Artavanis-Tsakonas, 1994). Consistent with the requirement for *N* in wing margin formation and growth, decreased *Su(H)* function results in a small third instar wing pouch (Schweisguth and Posakony, 1992) and very small adult wings, reminiscent of *vg* mutant wings (Ashburner, 1982). *Su(H)* activity is antagonized by *Hairless* (*H*), which encodes a novel nuclear protein (Bang and

Posakony, 1992). Indeed, loss of *H* function leads to the opposite phenotype, an abnormally large wing pouch (Bang et al., 1991). Thus it appears that multiple elements of the Dl-N signaling pathway operate during wing margin formation.

wg has been shown to interact with *N* during wing margin formation, indicating that these two genes function in the same pathway (Couso and Martinez Arias, 1994; Hing et al., 1994). Couso and Martinez Arias (1994) proposed several molecular models to explain this interaction, favoring a model in which N functions as a receptor for wg. The observations that *N* is required for *wg* expression (Rulifson and Blair, 1995) and that ectopic N and Dl induce *wg* demonstrate that *wg* is a downstream target of the N/Dl signaling pathway; however, we cannot eliminate the possibility that *wg* also functions upstream of *N* earlier during wing development.

Adult wing outgrowth requires factors expressed at the A/P compartment boundary

Our ectopic Dl expression experiments indicate that *Dl* can be sufficient to induce wing margin formation. We have strong evidence that the effects of ectopic Dl expression reflect the function of *Dl* during wild-type development. The UAS-Dl construct produces protein that is localized to the plasma membrane and cytoplasmic vesicles in a manner indistinguishable from wild-type Dl, and UAS-Dl rescues the *Dl* loss of function neurogenic phenotype in the embryo when driven by hairy-G4 (see Methods). Further, the ectopic expression phenotype is opposite to the loss of function phenotype and requires normal activity of N, the only known receptor for Dl.

We have found that ectopic expression of Dl near the A/P compartment boundary results in adult wing tissue outgrowth. Ectopic expression at other locations in the wing pouch causes ectopic gene expression and outgrowth of

wing disc tissue, but not adult wing tissue outgrowth. *Dl*-induced adult outgrowth is always associated with wing margin structures, indicating that disc tissue overgrowth may resolve unless it is maintained by an established wing margin. Distal outgrowth caused by *fng*⁻ clones also occurs only near the A/P boundary (Irvine and Wieschaus, 1994). It is therefore likely that factor(s) specific to the A/P border are required for induction of an ectopic wing margin and the resulting wing outgrowth. One candidate is *decapentaplegic* (*dpp*) which is expressed along the A/P border (Posakony et al., 1990). Ectopic *dpp* expression induces growth and pattern duplication in the wing (Capdevila et al., 1994). Moreover, overlapping expression of *wg* and *dpp* in the leg disc is required for proximodistal growth (Struhl and Basler, 1993). Ectopic Dl-induced growth cannot be due simply to the ectopic expression of *wg* in *dpp* expressing cells along the A/P boundary, because neither Dl-induced *wg* expression in the ventral compartment, nor ptcG4 driven *wg* expression in both compartments is sufficient to cause adult wing outgrowth(Figure 3.3A and E. Wilder, pers. communication). It appears that the constellation of ectopic gene expression induced by ptcG4-Dl acts in concert with endogenous factors at the A/P compartment boundary to cause ectopic wing outgrowth. It will be interesting to determine how endogenous factors at the D/V and A/P boundaries interact to cause growth of the wing.

Delta can inhibit signal reception by Notch in the same cell

Ectopic Dl expression induces *wg* and *cut* only at the borders between Dl-expressing and non-expressing cells, and not in all cells that express ectopic Dl. One possible explanation for this observation is that Dl inhibits N receptor activity when expressed within the same cell as N; Irvine and Wieschaus (1994) have proposed an analogous model for *fng* and its putative receptor. In our

model, the ratio of Dl to N within a cell would determine its ability to receive a signal via N. Signaling would only occur when cells with a Dl/N ratio low enough to allow signal reception are juxtaposed to Dl expressing cells. This model explains why there is decreased Dl signaling within the ptcG4-Dl stripe. We have tested three predictions of this model: 1) Signaling should be strongest between cells that express high levels of Dl and cells which express low levels of Dl. Indeed, we observe maximal *wg* and *cut* induction immediately posterior to the ptcG4-Dl stripe, where cells expressing high Dl levels are juxtaposed to cells expressing low levels. 2) Increasing the level of N should relieve the Dl-mediated inhibition of N receptor activity. As expected, in discs expressing N and Dl under the control of ptc-G4, *cut* is expressed throughout the width of the ptcG4 stripe. 3) Expressing high levels of Dl should mimic reduction in *N* function. In fact, this prediction is met by two paradoxical observations. Dl overexpression in the wing blade results in hypertrophy of wing veins (Figure 3.3A-B), and Dl overexpression in the proneural cluster results in the development of extra sense organs; both of these phenotypes are similar to those caused by reduction in *N* or *Dl* activity (de Celis and Garcia-Bellido, 1994; Parks and Muskavitch, 1993; Figure 3.1C-D; D. Doherty and G. Feger, unpublished).

Dl could exert its inhibitory effect on N activity by directly interacting with N or by indirectly inhibiting N activity via other proteins. Alternatively, Fehon et al. (1990) proposed that Dl in receiving cells might interfere with N signaling by binding Dl ligand on the signaling cell(s). It should be noted that inhibition of signal reception by Dl does not appear to play a role in the early D/V patterning of the wing, because we did not detect a sharp border between *Dl* expressing and non-expressing cells; however, later, the *wg* stripe is flanked on both sides by *Dl* expressing cells, and signaling from *Dl*-expressing to non-expressing cells may be important.

A model for symmetrical gene activation at the D/V boundary

N activity is required on both sides of the D/V boundary for wing margin formation, while *Dl* and *Ser* are each required only on the ventral and dorsal sides respectively. In addition, cells in the dorsal and ventral compartments respond differently to ectopically expressed Ser and Dl, while cells in both compartments respond equally to ectopically expressed activated N (Table 3.2). One possible explanation for the different activities of *Dl* and *Ser* could be their roles as compartment-specific signals. Bi-directional signaling between dorsal and ventral compartment cells has been invoked as a mechanism to generate the wing margin and symmetric growth of the wing (Irvine and Wieschaus, 1994; Rulifson and Blair, 1995; Williams et al., 1994). Compartment-specific signaling can be generated by spatial restriction of the ligand or by spatial restriction of the response. For example, *fng* and *Ser* are expressed only in the dorsal compartment of second instar wing discs (Irvine and Wieschaus, 1994; cited in Kim et al., 1995). Further, *Ser* is ectopically induced at the borders of fng^- clones in the dorsal compartment, and ectopic *fng* induces *Ser* in the ventral compartment; however, ectopic *Ser* does not induce *fng*, indicating that *Ser* functions downstream of *fng*. Irvine and Wieschaus (1994) have proposed that *fng* encodes a compartment-specific dorsal to ventral signal, and Kim et al. (1995) have shown that *fng*-expressing dorsal cells can recognize when they are adjacent to cells not expressing *fng* and respond by activating expression of *Ser*, which they propose encodes a dorsal to ventral signal. The existence of a reciprocal signal from ventral to dorsal cells is based on the observation that cells both inside and outside fng^- or ap^- clones are transformed into wing margin (Irvine and Wieschaus, 1994; Williams, et al. 1994). The N receptor is required for signaling in both directions, because loss of *N* function on one side of the D/V boundary

eliminates *wg* expression and causes loss of wing tissue on both sides of the boundary (de Celis and Garcia-Bellido, 1994; Rulifson and Blair, 1995).

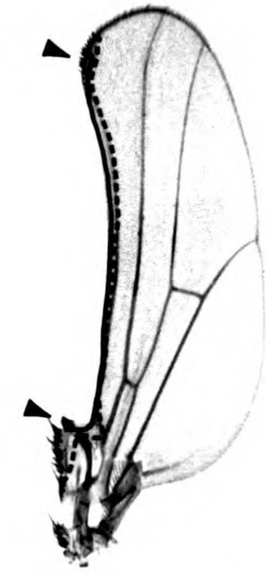
We propose that *Dl* acts as a ventral to dorsal signal which activates N to induce wing margin specific genes during second instar development. *Dl* displays three characteristics expected of such a signal: 1) *Dl* is required in ventral cells at the D/V boundary for wing margin formation. 2) *Dl* is expressed at the D/V compartment boundary in second instar discs. 3) Ectopic *Dl* can induce ectopic wing margin formation and wing outgrowth, but only in the dorsal compartment. For the *Dl* signal, restriction of the response plays an important role in compartment specificity. For *Ser*, restriction of the response as well as restriction of the ligand is important, since *Ser* is expressed only in the dorsal compartment, and only ventral cells respond to ectopic Ser.

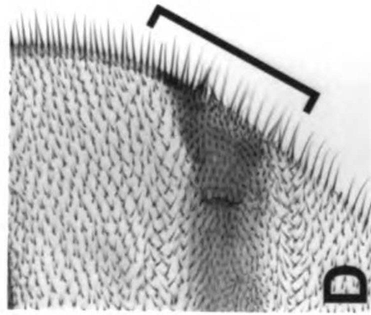
We propose the following model to explain the early steps of wing margin formation during the second larval instar (Figure 3.6). Ser in the dorsal compartment induces *wg* expression and *Dl* expression or activity in ventral compartment cells. In turn, *Dl* in ventral compartment cells signals back to dorsal compartment cells via *N* to induce *wg* and to reinforce *Ser* expression or activity (Figure 3.6). Indeed, preliminary results indicate that ectopic Dl expression induces *Ser* in the dorsal compartment of ptcG4-Dl discs (C. Micchelli, personal communication) and that ectopic Ser induces *Dl* in the ventral compartment of ptcG4-Ser discs (D. Doherty and G. Feger, unpublished). Kim et al. (1995) have shown that *fng* acts upstream of *Ser* to activate *Ser* expression in cells that do not express *fng*. The role of *fng* could be to initiate the positive feedback loop between *Dl* and *Ser* either by signaling from dorsal to ventral cells to activate *Dl* in the ventral cells, or by inhibiting the response to *Ser* and activating the response to *Dl* in dorsal cells, creating a border for *Dl* and *Ser* signaling, or both. As wing development proceeds, the early pattern of *Dl*

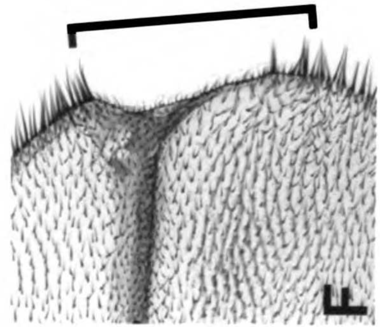
expression evolves into the third instar pattern where *Dl* is expressed in two stripes flanking the *wg*-expressing cells at the D/V boundary. It is likely that the combined activities of *wg, Dl, Ser* and other genes generate the later expression pattern. This system enables an intrinsically asymmetric boundary between *ap* expressing and non-expressing cells to induce the symmetric patterns of growth and gene expression required to form the wing.

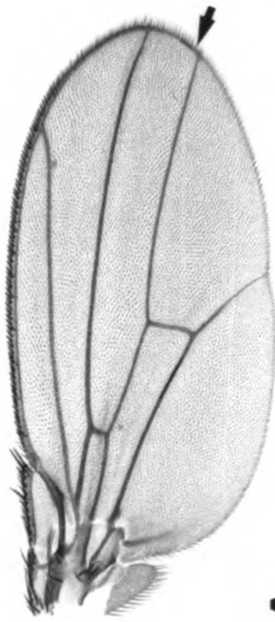
Conservation of mechanisms for axis formation and distal outgrowth in appendage development

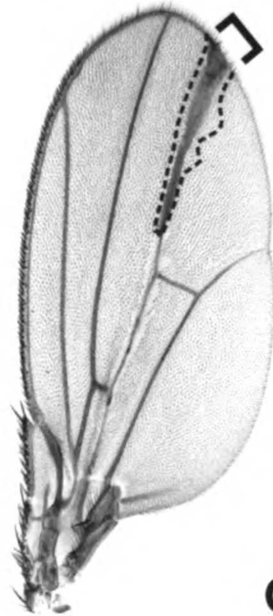
How axes are specified is a universal problem during appendage development. Data from a variety of species suggest that there may be a limited number of molecular mechanisms for generating axes and other pattern information. For example, the *hedgehog* pathway is used to pattern different types of appendages in animals as evolutionarily divergent as the fly, chicken and mouse (for review see Perrimon, 1995; Tabin, 1995). The *Dl-N* signaling pathway described in this paper may also be used in vertebrate limbs. *Jagged,* a murine member of the *Dl/Ser* family, is expressed in developing limbs (Lindsell et al., 1995). *Wnt-7a,* a mouse homolog of *wg,* has been shown to function in D/V patterning of limbs (Parr and McMahon, 1995; Riddle et al., 1995). It remains to be determined whether the *Dl-N/Ser-N* signaling pathways are used for limb axis formation throughout the animal kingdom.











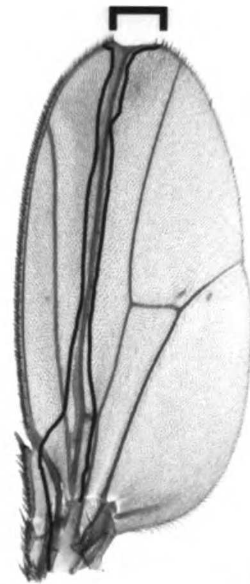


Figure 3.1 Ventral but not dorsal Dl^{rev10} clones that abut the D/V boundary cause loss of wing margin.

All wings are oriented with anterior up, proximal to the left. Dashed lines mark clone boundaries in the dorsal compartment, while solid lines mark clone boundaries in the ventral compartment.

(A) Unirradiated control f^{36a}; Dl^{rev10} *e/bld cu* f^{+98B} wing. The A/P compartment boundary between veins 3 and 4 is marked by an asterisk.

(B) Wing with a Dl^{rev10} that crosses the D/V boundary, the anterior wing margin and a large part of the wing blade are missing. Black arrowheads mark groups of *forked ebony* bristles at the clone borders.

(C) Wing with a Dl^{rev10} clone that abuts the D/V boundary from the dorsal side (bracket), while the dorsal marginal hairs are missing, the wing margin and blade are intact.

(D) High magnification view of (C).

(E) Wing with a Dl^{rev10} clone that abuts the D/V boundary from the ventral side (bracket), there is a gap in the wing margin and adjacent wing blade.

(F) High magnification view of (E).

Table 3.1 *Dl* is required only in the ventral compartment for wing margin formation

location of clone◊	total clones§	only dorsal bristles absent	only ventral bristles absent	dorsal and ventral bristles absent (no gap)	gap in margin and blade
dorsal	11	11	0	0	0
ventral	10	0	2†	0	8
dorsal and ventral	31	0	1‡	2	28
internal (dorsal or ventral)	14	0	0	0	0

◊ clone location was determined based on presence of *f* hairs adjacent to the D/V boundary in the dorsal, ventral or both compartments; *f* hairs were confined to the wing blade in internal clones

§ only wings with a *Dl* wing vein phenotype were analyzed; 6 wings with clones were unscorable due to gross distortion of the wing tissue

† wings with clones that appear to abut the boundary because they eliminate ventral bristles

‡ wing with both dorsal and ventral *forked* hairs, wing margin and dorsal bristles unaffected

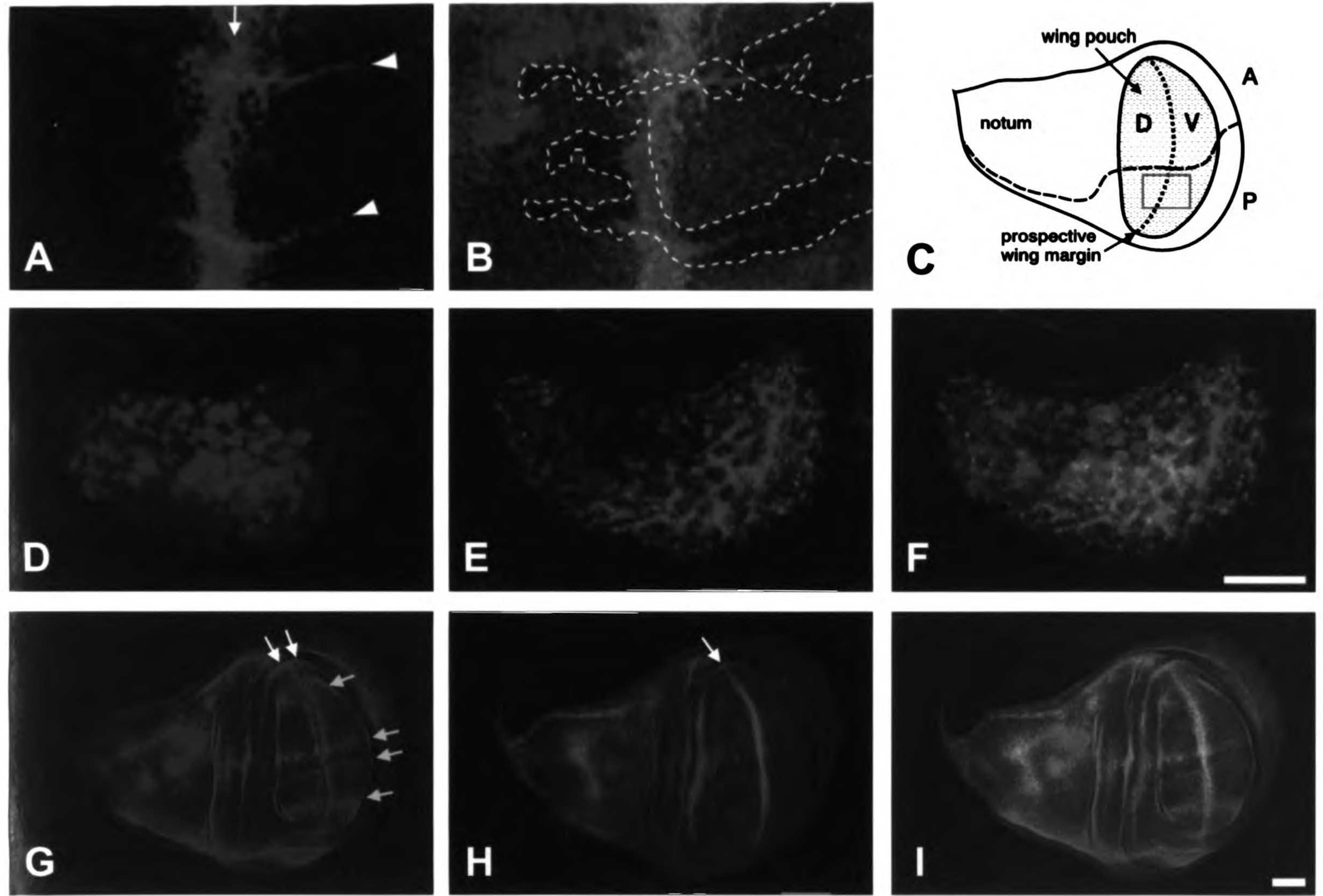


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Figure 3.2 *Dl⁻* clones and wild-type Dl expression in the wing disc.

(A-B) Homozygous Dl^{rev10} clones in a third instar wing disc (box in (C) marks the region of the disc in the images)

(A) wg expression (red). The arrow marks the endogenous stripe of wg expression at the D/V boundary, while the arrowheads mark ectopic wg expression.

(B) Overlay of (A) with the myc epitope tag signal (green); the homozygous Dl^{rev10} clones (dotted lines) are marked by the absence of myc signal. Ectopic *wg* is present approximately one cell width from the nuclei of myc-expressing cells.

(C) Fate map of wing disc (Bryant, 1975) adapted from Diaz-Benjumea and Cohen (1993); the box marks the region shown in (A-B).

(D-F) Dl protein and ap-lacZ expression in a mid-second instar wing disc. Dorsal is to the left. Scale bar represents 8 μm.

(D) ap-lacZ (green) is expressed in the nuclei of dorsal compartment cells.

(E) Dl protein (red) is expressed in the membranes of cells at the D/V boundary and mainly in the ventral compartment.

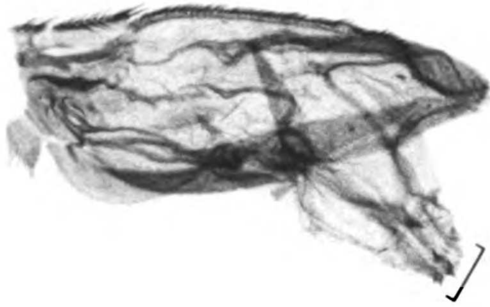
(F) Overlay of (D) and (E)

(G-I) Dl and wg expression in a wild-type third instar wing disc. In all wing disc figures anterior is up, dorsal is to the left. Scale bar represents 50 μm.

(G) Endogenous Dl (green) is expressed in two stripes of cells along the prospective wing margin (white arrows), the prospective wing veins (yellow arrows) and in proneural clusters.

(H) Endogenous wg (red) is expressed in the prospective wing margin (white arrow) and in a band across the notum.

(I) Overlay of (G) and (H). The wingless stripe is flanked by the Dl stripes.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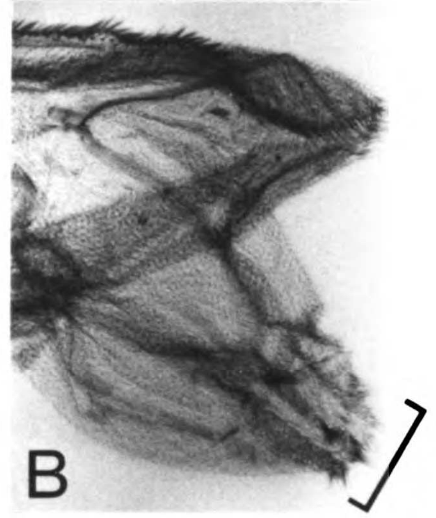
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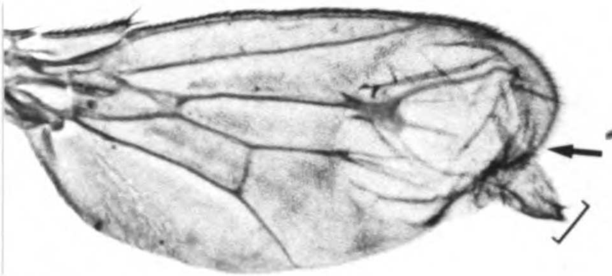
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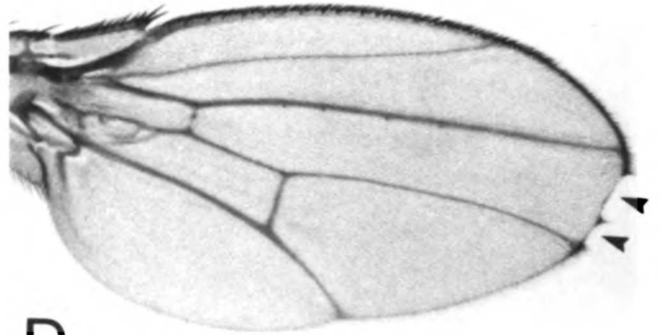
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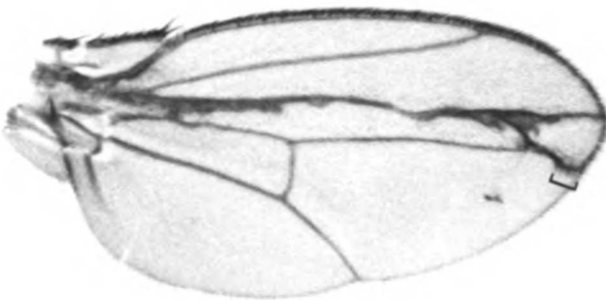
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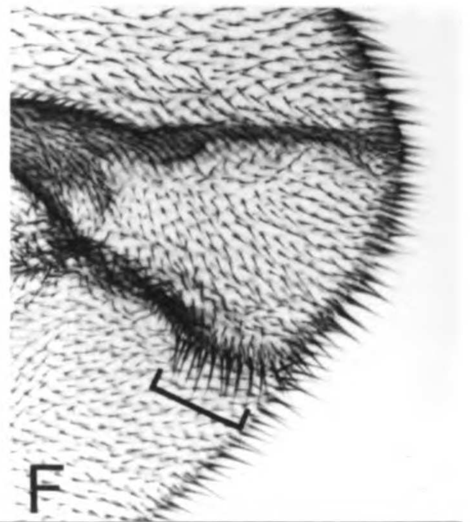
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Figure 3.3 Ectopic wing margin formation and wing tissue outgrowth induced by ectopic Dl is suppressed by N^{ts}

(A) Wing from a ptcG4-Dl fly shifted to 29°C for 24hr during the late second and early third larval instars. Wing outgrowth with hairs characteristic of the wing margin always occurs on the dorsal side of the wing (bracket).

(B) High magnification view of (A), showing the hairs at the distal tip of the outgrowth.

(C) Wing from a N^{ts}/yw; ptcG4/+; UAS-Dl/+ female shifted to 29°C for 24hr during the late second and early third larval instars. Note the *Dl*-induced outgrowth with hairs characteristic of the wing margin at the tip (bracket).

(D) Wing from a N^{ts}/Y; ptcG4/+; UAS-Dl/+ male shifted to 29°C for 24hr during the late second and early third larval instars. The *Dl*-induced ectopic outgrowth is completely suppressed (compared to (C)). Notches in the distal wing (arrowheads) are due to the reduction in N activity (see text).

(E) Wing from a ptcG4-N fly shifted to 29°C for all of the second and third larval instars. Ectopic margin-like hairs along vein 4 (bracket) occur on both the dorsal and ventral sides of the wing blade.

(F) High magnification view of (E), showing ectopic hairs characteristic of the wing margin (bracket).

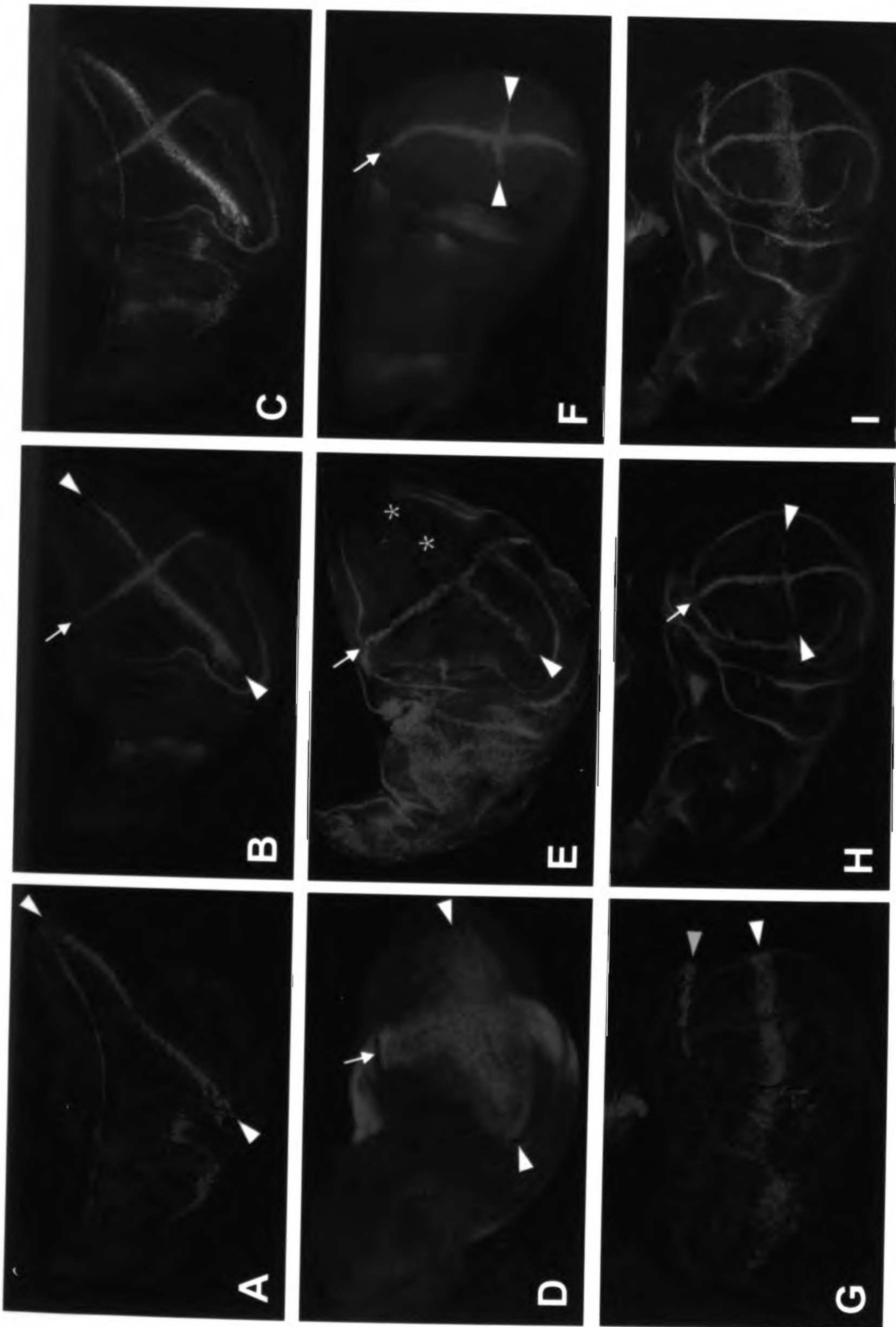
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Figure 3.4 Ectopic Dl or N expression induces wg, cut and vg in the third instar wing disc.

Anterior is up and dorsal is left in all images.

(A-E) Dl, wg, vg, and cut expression in a ptcG4-Dl third instar wing disc. Ectopic Dl induces wg, vg, and cut expression as well as overgrowth of the dorsal and ventral wing pouch. UAS-Dl was expressed using the ptcG4 enhancer trap in larvae raised at 29°C.

(A) Dl (green) is expressed ectopically in a stripe along the A/P compartment boundary (white arrowheads). Endogenous Dl expression is barely visible flanking the D/V boundary at this contrast setting.

(B) wg (red) is induced ectopically in a stripe along the A/P compartment boundary (white arrowheads). Endogenous wg expression is visible in the D/V compartment boundary cells (white arrow).

(C) Overlay of (A) and (B).

(D) vg (red) is induced ectopically in a wide band centered on the A/P compartment boundary (white arrowheads). Endogenous *vg* expression is visible in a wide band of cells centered on the D/V boundary (white arrow).

(E) cut (red) is induced ectopically in a stripe along the A/P compartment boundary only in the dorsal compartment (white arrowhead). Asterisks mark the A/P boundary in the ventral compartment which is devoid of cut expression.

(F-I) N, wg and cut expression in a ptcG4-N third instar wing disc. Ectopic N induces wg and cut expression. UAS-N was expressed using the ptcG4 enhancer trap in larvae raised at 29°C.

(F) wg (red) is induced ectopically in cells along the A/P compartment boundary only near the prospective wing margin (white arrowheads). Ectopic wg is expressed in cells on both the dorsal and ventral sides of the wing margin.

Endogenous wg expression is visible in the wild-type wing margin (white arrow).

(G) N (green) is expressed ectopically in a stripe along the A/P compartment boundary (white arrowheads). The yellow arrowhead marks ptcG4-N expression in the peripodial membrane.

(H) cut (red) is induced ectopically only in cells along the A/P compartment boundary near the wing margin (white arrowheads). Ectopic cut is expressed in cells on both the dorsal and ventral sides of the wing margin. Endogenous cut expression is visible in the wild type wing margin in the same cells that express wg (white arrow).

(I) Overlay of (G) and (H). cut is expressed within the ptcG4-N stripe. There is only slight overlap (yellow) because N is a transmembrane protein while cut is nuclear.

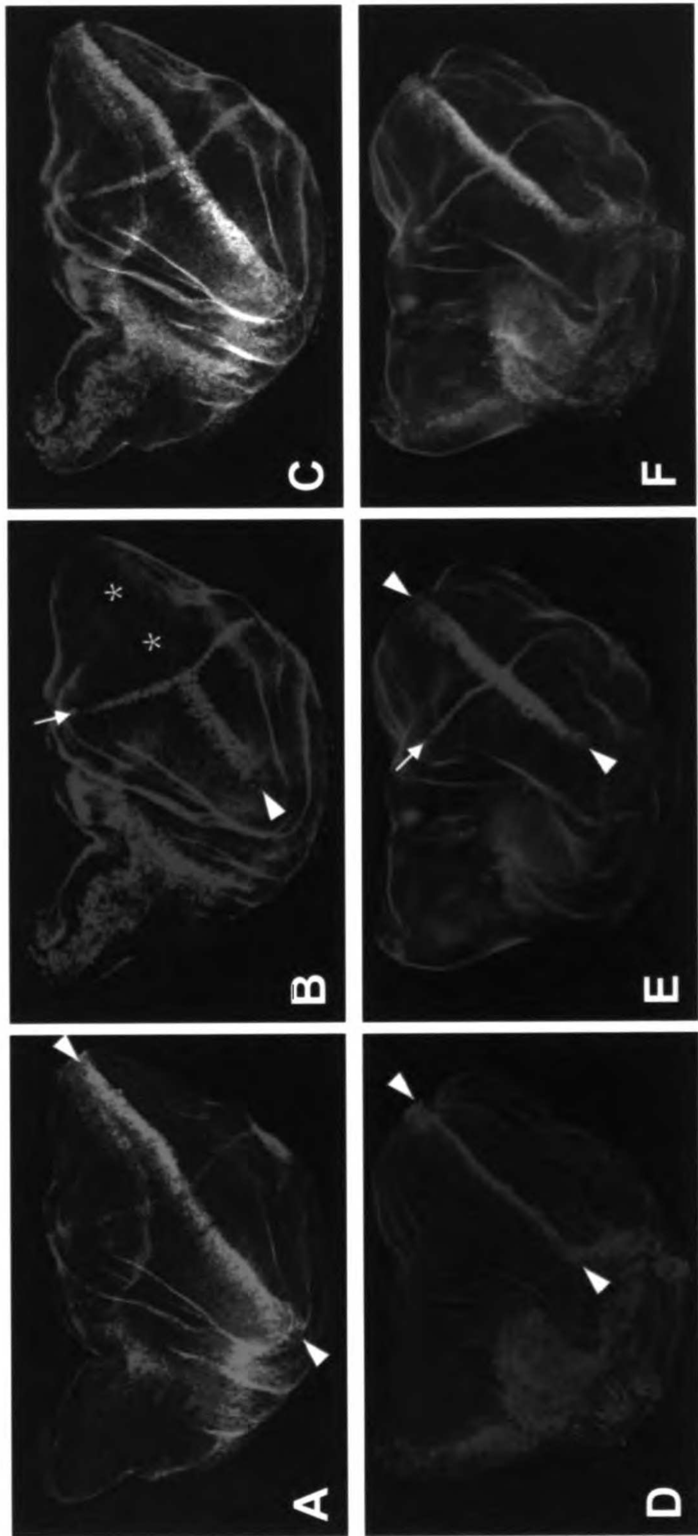
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Figure 3.5 cut expression in ptcG4-Dl+N and ptcG4-activated N third instar wing discs.

(A-C) Dl, N and cut expression in a ptcG4-Dl+N third instar disc. Ectopic N and Dl together still induce cut expression only in the ventral compartment. UAS-Dl and UAS-N were expressed using the ptcG4 enhancer trap in larvae raised at 29°C.

(A) Dl (blue) and N (green) are expressed ectopically in a stripe along the A/P compartment boundary (white arrowheads).

(B) cut (red) is induced ectopically within the ptcG4 stripe only in the dorsal compartment (white arrowhead). Asterisks mark the A/P boundary in the ventral compartment which is devoid of cut expression.

(C) Overlay of (A) and (B). Ectopic Dl, N and cut expression overlap in the dorsal compartment (white signal), but not in the ventral compartment (blue signal).

(D-F) N and cut expression in a ptcG4-activated N third instar disc. Ectopic activated N induces cut expression in the dorsal and ventral compartments. UAS- activated N was expressed using the ptcG4 enhancer trap in larvae raised at 16°C.

(D) activated N (green) is expressed ectopically in a stripe along the A/P compartment boundary (white arrowheads).

(E) cut (red) is induced ectopically within the ptcG4 stripe in both the dorsal and ventral compartments (white arrowheads). Endogenous cut expression is visible at the D/V boundary (white arrow).

(F) Overlay of (D) and (E). Ectopic activated N and cut expression overlap in the dorsal and ventral compartments.

Table 3.2 Effects of ectopic Dl and Ser expression in the dorsal and ventral compartments.

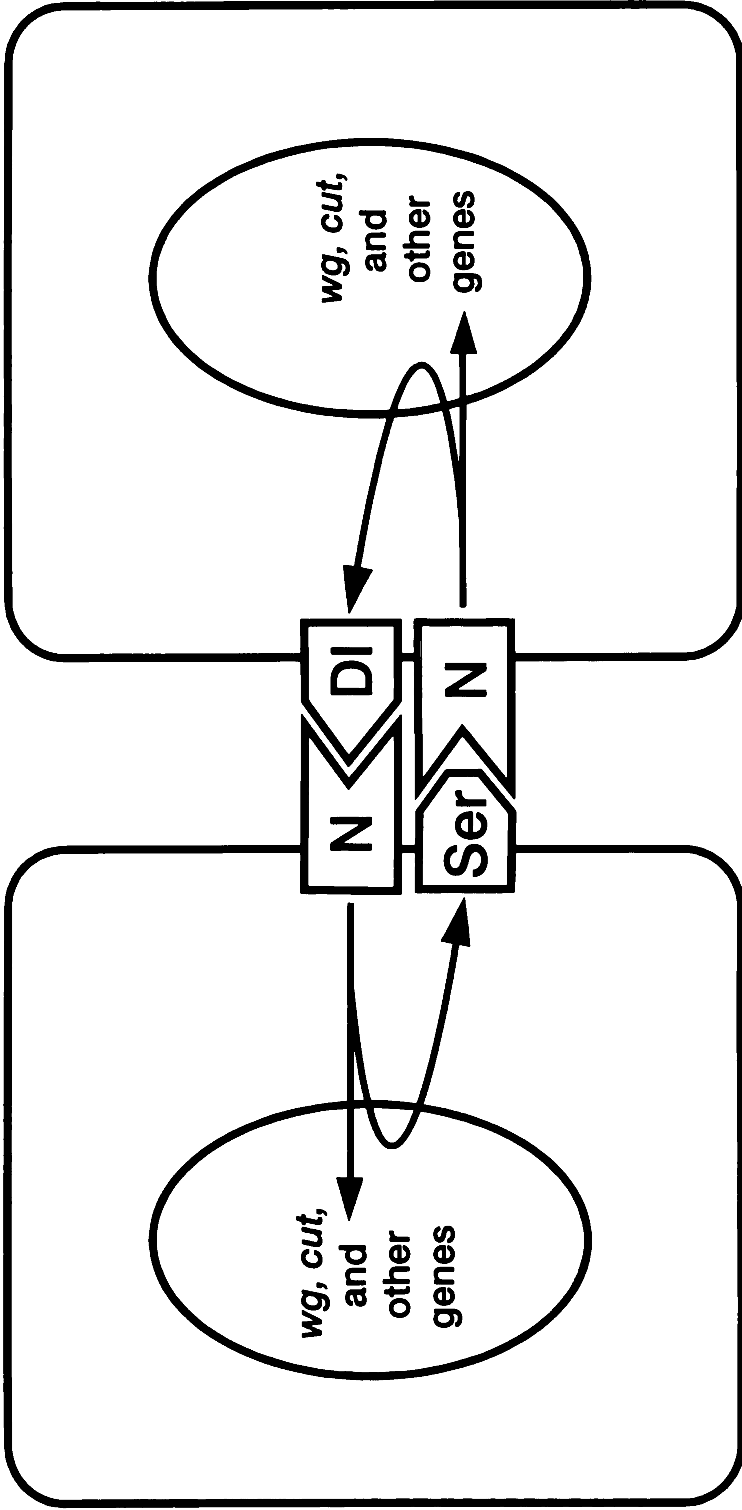
		ptcG4-Dl	ptcG4-actN	ptcG4-Ser
DORSAL	Gene expression:			
	wg	++	++	- (2)
	vg	++	++	- (2)
	cut	++	++	- (3)
	Disc outgrowth	++	++	- (1) (2)
	Adult outgrowth	++	N.D.*	- (1) (2)
	Wing margin	++	N.D.*	- (1) (2)
VENTRAL	Gene expression:			
	wg	+	++	++ (2)
	vg	++	++	++ (2)
	cut	-	++	++ (3)
	Disc outgrowth	++	++	++ (1) (2)
	Adult outgrowth	-	N.D.*	++ (1) (2)
	Wing margin	-	N.D.*	++ (1) (2)

* not determined because ptcG4-actN causes early pupal lethality

(1) Speicher et al. (1994)

(2) Kim et al. (1995)

(3) D. Doherty and G. Feger, unpublished



VENTRAL CELLS
DORSAL CELLS
wg, cut, and other genes
wg, cut, and other genes
Dl
N
N
Ser

Figure 3.6 Model for symmetrical gene expression at the D/V boundary mediated by a positive feedback loop.

Dl signals from ventral to dorsal cells via N to activate or maintain *Ser* and *wg* expression, while Ser signals from dorsal to ventral cells via N to activate or maintain *Dl* and *wg* expression. As wing development proceeds, the early pattern of *Dl* and *Ser* expression evolves into the third instar pattern where *Dl* and *Ser* are expressed in two stripes flanking the *wg*-expressing cells at the D/V boundary. It is likely that the combined activities of *wg, Dl, Ser* and other genes generate this later expression pattern.

CHAPTER 4

CONCLUSION

PART I

Is *bib* in the neurogenic gene pathway?

It has been assumed that *bib* functions in parallel to the other neurogenic genes on the basis of negative genetic results (Campos-Ortega and Jan, 1991). We present suggestive evidence that *bib* does in fact function together with the other neurogenic genes in the *N* pathway. *bib* is expressed in nearly all tissues that require NG gene function. bib protein colocalizes with Dl and N, indicating that it functions in the same subcellular locations as these proteins. *bib* is required for proper signal reception, and is not required to send the signal. Finally, we have intriguing preliminary evidence demonstrating that *bib* and *Dl* can function synergistically. Our data do not however exclude models in which *bib* is required for the proper reception of a second lateral inhibition signal from the presumptive neural precursor cell (NP), or even for reception of an as yet unknown signal that would mediate interactions between the presumptive epidermal cells but not with the NP.

We propose that *bib* facilitates lateral inhibition but is not an obligatory component of the signaling pathway. This model can explain why the loss of function *bib* phenotype is often weaker than that of the other neurogenic genes, since it would not be absolutely required to receive the signal, but rather to amplify it. The severity of the *bib* mutant phenotype seems to correlate with the efficiency of signaling that might be required for a particular process. Efficient signaling is presumably required during the rapid segregation of neuroblasts in the embryo, and loss of *bib* function causes a severe phenotype where many proneural cluster cells adopt the neural fate. Segregation of both embryonic and adult SOP's in the peripheral nervous system is a more leisurely process, and correspondingly, the *bib* mutant phenotype in the PNS is less severe; there is only a two-fold increase in SOP's. An extreme example is in the differentiation steps

of the SOP. *bib* is not expressed at all in the developing adult sense organ, where pairs of cells interact over the course of several hours to determine their cell fates. *bib* is however capable of augmenting neurogenic gene pathway activity in this lineage; ectopically expressed bib mimics the effects of ectopically expressed N. This type of model makes the prediction that changes in *bib* activity should be able to modify the phenotypes caused by gain or loss of function in the other neurogenic genes, *i.e.* genetic interaction. So far, the only example of interaction is the synergistic phenotype caused by ectopic expression of bib and Dl in the sense organ lineage. Ectopic bib expression or loss of *bib* function might also modify the phenotypes caused by overexpression of the other neurogenic genes in the sense organ lineage and other tissues. Knowing more about the biochemical activity of *bib* and what proteins *bib* contacts during its function will make it possible to design more specific tests of how *bib* might affect the activity of the neurogenic signaling pathway.

One could imagine a variety of mechanisms by which bib could augment activity of the neurogenic gene pathway. bib could be part of the Dl/N complex acting to enhance the binding between N and Dl or to increase the response generated by N as a result of Dl binding. Alternatively, bib could concentrate signaling components in particular regions of the plasma membrane, allowing efficient interaction between ligand and receptor. The ß-catenin homolog, armadillo, provides a link between the *wg* signaling pathway and cell adhesion at aderens junctions (Peifer et al., 1993). bib could provide a similar link for the neurogenic gene pathway, as both bib and N are concentrated at the position of adherens junctions in the neuroectoderm (Figure and (Fehon et al., 1991).

Which bib domains are required for activity?

The N-terminal half of *bib* has intriguing sequence similarity to channel proteins, while the C-terminal half has no similarity to known proteins (Figure and(Reizer et al., 1993). A first step to understanding *bib* function would be to determine the significance of the channel domain by making mutant forms of *bib* and testing them in the in vivo assay for *bib* activity described in Chapter 2. Testing the activity of N-terminal "channel-only" and C-terminal "tail" constructs (Figure), as well as point mutations in the channel forming domain should reveal whether a functional channel domain is required for *bib* activity. We have subcloned one myc-marked N-terminal construct into a UAS vector and transformed it into flies. In *bib* mutant embryos expressing the transgene, we detected low levels of truncated bib protein in the plasma membrane; however, the construct displayed no rescuing activity. While this preliminary data indicates that the channel domain is not sufficient for function, it will be important to look at a variety of mutant forms of bib expressed at higher levels.

What is the biochemical activity of bib?

The similarity of bib to channel proteins, indicates that bib may function as a channel. Other MIP family members have been shown to function as tetramers, so bib may have intrinsic channel activity, or it may complex with other channels to regulate their activity. Many channel proteins consist of subunits that form the pore and separate ß subunits that influence the activity or regulation of the channel. The long C-terminal domain of bib may function like a ß subunit, regulating the activity of the potential bib channel or other similar family members. Further study of *bib* will add to our understanding of how a channel protein might influence cell fate. The only other example where a channel protein may affect cell fate determination comes from the weaver

mutation in the mouse. The mutation is a single base-pair change in the *Girk2* gene that encodes an inward rectifying potassium channel, causing granule cells in the cerebellum undergo apoptosis (Patil et al., 1995).

It is possible to assay a protein for water channel activity using a permeability assay in Xenopus oocytes (Preston et al., 1992). While our preliminary experiments in cooperation with Verkman laboratory (U.C.S.F.) have failed to demonstrate water channel activity, we have not determined whether our *bib* constructs are actually expressed in the oocyte, so a more comprehensive study is in order. The full-length *bib* gene could be tested for activity, as well as truncated forms consisting of the channel domain alone. In addition, the possibility that bib regulates other channels could be explored by coexpressing *bib* with other MIP family members and looking for changes in channel activity and specificity.

A second system for assaying water channel activity in *Drosophila* Malpighian tubules has been developed (Dow et al., 1994). With this assay, UAS-bib constructs can be tested for intrinsic channel activity and for effects on the activity of other channels in a context where it is normally expressed.

S2 cells provide another system for testing for bib activity. (Fehon et al., 1990) has shown that N and Dl bind to each other and that Dl is endocytosed in a N-dependent manner. Co-labelling transformed cells with antibodies to N, Dl and bib will test whether bib is colocalized with the N/Dl complex. A more functional assay has been developed by (Fortini and Artavanis-Tsakonas, 1994) , in which Su(H) translocates to the nucleus in response to Dl binding N; it is possible that expression of *bib* in the *N*-expressing cells could enhance the transport of Su(H) to the nucleus.

Does bib physically interact with other proteins?

Demonstrating that the *bib* cDNAs are functional has opened up a wide variety of potential experiments to look for physical interactions. The long C-terminal domain of *bib* is predicted to be cytoplasmic and is ideal for use in the yeast two-hybrid system to look for proteins that physically interact with bib (Chien et al., 1991). The interaction trap system has been used to demonstrate a physical interaction between N and both the *Su(H)* and *dx* gene products (Diederich et al., 1994; Fortini and Artavanis-Tsakonas, 1994).

There is evidence for a physical interaction between bib and the extracellular domain of N. M13 phage which express bib are enriched from a library of phage by binding to Schneider cells that express the extracellular domain of N (C. Wesley and M. Young, personal communication). It will be interesting to test for physical association between bib, N, Dl and the other neurogenic gene products using the assays described above and using more conventional biochemical methods like immunoprecipitation, taking advantage of the anti-bib antibody described in this work.

Does *bib* interact with other genes?

The data we have presented in Chapter 2 suggest that instead of operating in parallel to the other neurogenic genes, *bib* functions in the same genetic pathway. The most important evidence is the synergistic interaction between ectopically expressed *bib* and *Dl* in the adult sense organ. This is an intriguing observation and needs to be followed up in depth. The phenotypes caused by ectopic expression of *bib* in combination with the other neurogenic genes may reveal further interactions. The mainstay of genetic approaches for finding genes which function together has been to look for dominant second site mutations that enhance or suppress the phenotype of known adult viable mutations. Attempts

to generate viable alleles through imprecise excision of P-element alleles have failed, as have attempts to generate deficiencies of the *bib* genomic region (unpubished results). In the absence of these more conventional tools, it is also possible to look for mutations that enhance or suppress the ectopic expression phenotype of a gene. While it should be easy to identify mutations that enhance the 109-68; UAS-bib phenotype in adults (described in Chapter 2), it may be impossible to identify suppressors of this subtle phenotype.

PART II

We have demonstrated that Dl is required in the ventral compartment for wing margin formation. In addition, ectopic Dl expression can induce wing margin formation, and cells in the dorsal and ventral compartments respond differently to Dl. In light of these findings, we have proposed that *Dl* encodes a ventral to dorsal signal, and that Dl and Ser create a positive feedback loop via Notch to generate symmetrical gene expression and growth centered on the D/V boundary. During wing development, *N* pathway activity is modulated in a position-dependent manner by restricted expression of the ligand Ser, and by compartment-specific differences in the response of N to its ligands. Furthermore, there is emerging evidence that *N* pathway signaling is integrated with inputs from other signaling pathways to generate outgrowth and wing margin formation. In this section, I will discuss a number of issues raised by this work to be addressed by future research.

What is the mechanism for the compartment-specific response?

Compartment-specific signaling by Dl and Ser cannot be explained by restriction of the ligands alone, because *Dl* is expressed throughout the wing pouch, and more importantly, the two ligands have different effects when ectopically expressed in the same compartment. Furthermore, the compartment-specificity is probably not due to differences in signal transduction events downstream of N activation, because expression of a constitutively activated form of N induces equal effects in both compartments. Therefore, the mechanism that generates the compartment-specific response must impinge on the extracellular domain of N and/or the two ligand molecules. Any mechanism that confers compartment-specificity on the N-ligand complex must require asymmetric expression of some gene product(s). *ap*, *fng* and *Ser* are all potential

mediators of compartment-specificity because they are all required for wing margin development and they are specifically expressed in the dorsal compartment. Asymmetric expression of these upstream genes could be translated into the compartment-specific responses to Dl and Ser using at least two different mechanisms: 1) direct binding of the compartment-specific component to N or the ligands; 2) compartment-specific modification of N or the ligands (covalent modification, proteolysis, etc.). There is preliminary evidence for a physical interaction between putatively secreted fng protein and the extracellular domain of N. M13 phage which express fng are enriched from a library of phage by binding to Schneider cells that express the extracellular domain of N (C. Wesley and M. Young, personal communication). Therefore fng is a leading candidate for a factor that binds N to modulate its responsiveness to Dl and Ser.

Dl and Ser are transmembrane proteins which both contain EGF-like repeats and share significant homology in their extracellular domains. In the embryo, *Ser* expression can at least partially reduce the nervous system hypertrophy caused by lack of *Dl* (Gu et al., 1995). Although much of the difference between *Dl* and *Ser* function can be explained on the basis of their distinct expression patterns, there must be particular domains within the proteins that allow them to have compartment-specific effects in the developing wing. These domains could be identified by making chimeric UAS constructs consisting of different portions of the Dl and Ser genes, and expressing the constructs in discs to see which protein domains are required for activity in each of the two compartments. Once domains required for compartment-specificity are identified, they can be tested for physical interaction with N, fng and the other extracellular proteins known to function in wing margin formation. It will also be possible to identify proteins that bind these domains using any of a

number of methods including expression cloning, co-immunoprecipitation, and phage display selection (Smith, 1985). Although the developing wing is the first example where Dl and Ser have been found to send qualitatively different signals to the same cells, it is likely that they will do so in other tissues where they are both expressed.

Is bib involved in wing margin formation?

As described in Chapter 3, multiple *N* pathway genes are required for wing margin development. The neurogenic gene *big brain* (*bib*) may also have a function in transcompartmental signaling at the D/V boundary. *bib* is expressed at a high level in the *wg*-expressing cells at the D/V boundary in third instar discs (Figure 2.6G) and is induced ectopically in ptcG4-Dl discs (not shown); however, loss of *bib* function in cells at the D/V boundary does not cause loss of the wing margin (Figure 2.7D-F). In addition, ectopic expression of UAS-bib driven by *ptc*-GAL4 does not cause ectopic wing outgrowth, but we have not examined whether *bib* can induce *wg* or *cut*. The available evidence indicates that *bib* is not absolutely required to form the wing margin; further analysis will be needed to determine whether *bib* potentiates *N* signaling at the D/V boundary (see Chapter 2).

How is the *N* pathway integrated with other signaling pathways?

There are now multiple lines of evidence that input from the *N* signaling pathway can be integrated with inputs from other signaling pathways, such as the *wg* pathway. González-Gaitán and Jäckle (1995) have demonstrated that the *wg* pathway modulates the activity of the *N* pathway during the development of the stomatogastric nervous system. Moreover, E. Rulifson (personal communication) has used clonal analysis to show that *wg* and *disheveled* (*dsh*), a

downstream target of *wg*, are required to restrict *wg* expression to the narrow stripe of cells at the D/V boundary in the third instar wing disc. Interestingly, the expansion of *wg* expression observed in mitotic clones that lack *dsh* function is suppressed in clones that also lack *N* function, indicating that the expansion of *wg* is *N*-dependent. As we demonstrated in Chapter 3, the *N* signaling pathway induces *wg* expression; apparently, wg protein at the D/V boundary inhibits *N* pathway activity via *dsh* in the adjacent cells, restricting *wg* expression to a narrow stripe. Furthermore, *wg* expression at the D/V boundary is maintained by the flanking stripes of *Dl* and *Ser* expressing cells, and expression of *Dl* and *Ser* within these cells is dependent on *wg* (E. Rulifson, personal communication). Thus the *N* and *wg* pathways modulate each other to generate the highly refined pattern of gene expression and cell fates at the D/V boundary.

Is there cross-talk between the A/P and D/V patterning mechanisms?

Dorsal wing outgrowth caused by ectopic *Dl* expression and by *fng* mutant clones occurs only near the A/P boundary (Figure 3.3 and Irvine and Wieschaus, 1994). Wing veins and campaniform sensillae appropriate for this A/P location develop in these outgrowths. Correspondingly, the D/V axis appears relatively unaffected in the pattern duplications caused by perturbations in A/P patterning mechanisms. For instance, dorsal- and ventral-specific bristles form on ectopic wing margin induced by posterior compartment clones lacking *en* (Tabata *et al.*, 1995). The patterning mechanisms for one axis are able to induce the proper patterning in the other axis. Outgrowth is only generated when components specific to the D/V compartment boundary are expressed in proximity to components specific to the A/P compartment boundary; this also indicates that there is communication between the A/P and D/V patterning mechanisms. The observation that the neurogenic gene *groucho* prevents the

expression of *hh* and *en* along the D/V boundary in the anterior compartment (de Celis and Ruiz-Gómez, 1995), may be the first hint at the molecular basis for this communication. Generation of outgrowth in the wing provides another opportunity to study how inputs from multiple signaling pathways are integrated to determine cell fates.

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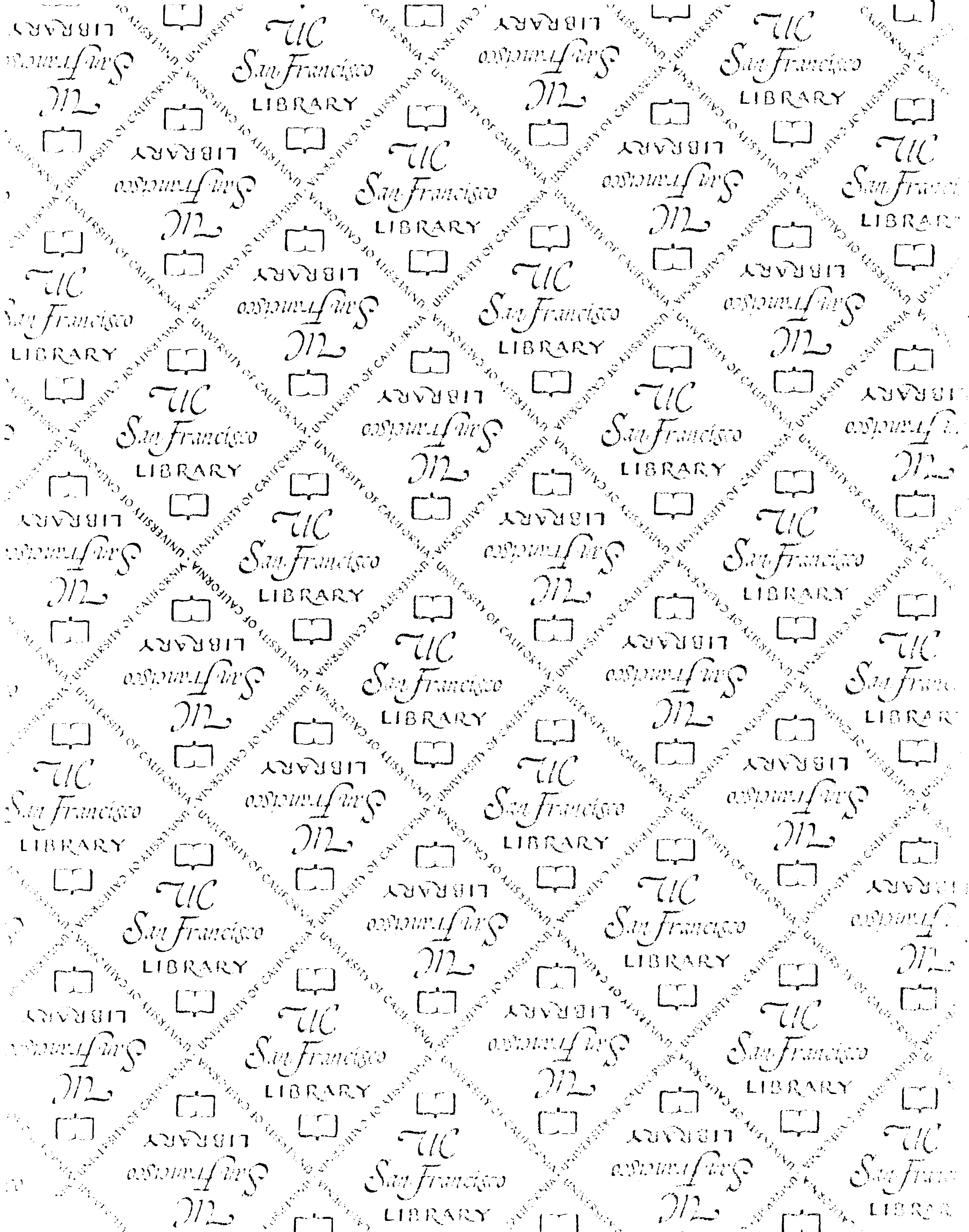
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