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HOW SISTER CELLS ACQUIRE DIFFERENT FATES:

THE ROLE OF NUMB IN ASYMMETRIC DIVISIONS

by

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DISSERTATION

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This dissertation is dedicated to my parents, Stan and Joanna Rhyu, who have loved and nurtured me over these twenty-eight years.

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Abstract

The four cells of an external sensory [es] organ, the neuron, its sheath cell, the hair and socket, are derived from three successive "asymmetric" divisions, in which daughter cells acquire different fates. The sensory organ precursor [SOP] divides first to form two secondary precursors, IIa and IIb, which undergo another round of division to create the four es organ cells. I determined by immunocytochemistry that numb is a membrane associated protein which localizes asymmetrically to one side of the predivisional SOP cell. During mitosis, numb segregates differentially to one daughter, presumably the IIb cell. In the *numb* loss of function mutant, the IIb cell differentiates as its sister cell, producing two IIa cells. Expression of numb in both the IIa and IIb during the time of SOP division results in the reciprocal transformation, suggesting that numb is required for IIb cell fate.

Mosaic studies and misexpression of numb during differentiation of the daughters of the IIa cell reveal numb is also required for hair cell fate. Involvement at this step reiterates the requirement for numb in multiple asymmetric fate decisions, enabling one cell to acquire a different fate than its sister. I examined the epistatic relationship between activated Notch and ectopic numb to discern how numb may impinge on Notch-mediated cell interactions. These data support a hypothesis whereby numb inhibits Notch activity in the IIb cell and the hair cell.

These experiments suggest that es cell fate determination occurs as three successive alternative fate decisions. The asymmetric distribution of numb confers an intrinsic difference between daughters in at least two and possibly all of these divisions. Localized numb expression is independent of the mesoderm and overlying epidermis, implying that localization reflects an intrinsic asymmetry in the predivisional cell.

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INTRODUCTION

Every multicellular organism begins as a single cell. How that first cell gives rise to two, then four, then eight, and eventually millions of cells which may have different morphologies and specialized functions is at the heart of the study of development. Two principal mechanisms have been forwarded for how cell diversity, or asymmetry, may be generated upon division: The first proposes an intrinsic difference between two daughter cells, by way of a differentially inherited factor or other distinguishing feature, while the second mechanism invokes communication by each daughter with its respective environment, leading to divergent paths of differentiation. Development of the external sensory [es] organ in the peripheral nervous system [PNS] of *Drosophila melanogaster* provides an opportunity to examine potential roles of both of these mechanisms in generating four distinctly differentiated cells. In this thesis, I present evidence that the intrinsic type of mechanism is utilized during the specification of the es organ cells. The goal of this work is to understand the role that lineage can play in generating diversity, as well as to explore how an inherited difference may be parlayed into cell fate determination. To provide a context for the results presented, I will begin with a discussion of the historical development of the notion of intrinsic asymmetry. I will next present some model embryonic systems where determinants are localized prior to division, contrasting these examples with our limited knowledge of asymmetry in post-embryonic divisions. In the final part of the Introduction, I will review the literature on differentiation of the es organ cells.

Intrinsic Asymmetry

historical review

Examples of "asymmetric" divisions abound in nature. Horvitz and Herskowitz (1992) have presented an extensive review of such divisions, distinguishing intrinsically asymmetric divisions from those in which initially identical daughter cells give rise to distinct cells upon interaction with the environment. The first observations of intrinsically asymmetric divisions were made in the latter part of the nineteenth century. This work by early embryologists and the century of conceptual theory preceding it laid the groundwork for modern studies of unequal divisions. The original work is described and analyzed in the classical text, The Cell in Development and Heredity (1925), by Wilson, while a thorough review with a modern perspective has been provided by Davidson (1986).

The marvel of how a simple, apparently uniform cell such as an egg could develop into a complex multicellular organism led Bonnet to postulate the preformationist theory in 1764. This theory proposed that each egg contained a complete but miniature form of the adult organism, and that fertilization would initiate its growth into adult proportions. The supposition that complexity was inherent in the egg predicted that development did not require generation of diversity. With the advent of microscopic experimentation, this model was dismissed as simplistic. Wolff (1759, 1768) toppled the premise of preformationists by demonstrating that chick blood vessels and gut arose from undifferentiated tissues. This first evidence of the epigenetic influence on diversity was followed by Pander (1817), von Baer (1828, 1837) and Kowalewsky (1867), who determined that skin, muscle and skeletal tissue types were derived from specific primitive germ layers which were themselves epigenetically formed.

Preformation was revisited, however, in a thoughtful proposal by Wilhelm His, who, in 1874, presented his *principle of organ-forming germ-regions*: "....every point in the embryonic region of the blastoderm must represent a later organ or part of an organ, and... every organ developed from the blastoderm has its preformed germ in a definite region of the germ disc..." His thus suggested that organs of the embryo may be preformed in the egg, but may not yet be recognizable. Lankester (1877) elaborated this theory, invoking precocious segregation of preformed molecules:

> "Though the substance of a cell may appear homogeneous under the most powerful microscope, it is quite possible, indeed certain, that it may contain, already formed and individualized, various kinds of physiological molecules. The visible process of segregation is only the sequel of a differentiation already established and not visible."

The development of this theory coincided with key lineage studies by Whitman, Rabl and Van Beneden. Whitman's analysis of cell lineage in the leech, *Clepsine* (1878), revealed that the cells of the ventral nerve cord entirely descend from a single pair of sister cells termed neuroblasts. Mesoderm and ectoderm were subsequently found to derive from small sets of mesoblast and ectoblast cells, respectively. This observation that the origin of certain tissues types could be traced to particular cells in the early embryo strongly supported the idea that the early embryo reflected a mosaic of predetermined cells.

How and at what stage of embryo cleavage were these areas delineated? Chabry (1887) and Roux (1888) were the first to address the equipotency of blastomeres at the two and four cell stages. Chabry, studying the ascidian egg, and Roux, working with frog eggs, independently concluded that the ablation of one or more cells at these stages resulted in the development of incomplete adult organisms: The missing portions corresponded to the blastomere(s) which had

been destroyed. Roux concluded that "the development of the frog-gastrula and of the embryo formed from it is, from the second cleavage onward, a mosaic-work, consisting of at least four vertical, independently developing pieces." This *mosaic theory of cleavage and development* was further supported by subsequent detailed lineage and cell disaggregation studies of Conklin (1897,1905), Wilson (1904) and others.

Although the mosaic behavior of blastoderm cells was becoming well established, an understanding of the physical basis for this behavior had progressed little beyond Lankester's proposed unrecognizable "physiological molecules." Roux (1883) and Weismann (1885) advocated a *Theory of Qualitative Nuclear Division*, which proposed that differentially segregated nuclear components accounted for the unique properties of individual cells. This theory was soundly disproven by two lines of experimentation: First, it was demonstrated that extrusion of part of the ooplasm of the unsegmented egg resulted in deformed larvae despite the presence of the intact nucleus. Moreover, application of mechanical stress to the dividing embryo caused the reorganization of its nuclei but did not affect normal development of the larvae. These findings suggested that key determinants for differentiation lay not in the nucleus, but in the ooplasm.

A further challenge to Roux's proposals was mounted by Driesch, whose observations appeared to contradict the basic tenets of *mosaic theory*. Driesch determined that a sea urchin blastomere isolated from a two, four, or, in some cases, eight cell embryo could develop into a miniature, though morphologically complete, larva. This result was subsequently found to be true for other animals (Wilson, 1893; Zoja, 1895; Morgan, 1895; Wilson, 1903), demonstrating that some early blastomeres are totipotent. Driesch concluded that "the [cells] produced by cleavage are completely equivalent and indifferent." He went on to

assert that early development was entirely directed by "regulative" interactions between cells.

The resolution of this conundrum was due first to the recognition that components of the cytoplasm are prelocalized. Ooplasm had been observed to be stratified, with a number of organisms displaying pigmented bands. In the most extensive work of this kind, Conklin recognized five distinctly pigmented areas in the ascidian, *Cynthia* (1905, 1912) and traced the lineages of these regions to their later development into five different tissue fates. This indicated that localized cytoplasmic components in the uncleaved embryo were correlated with differentiation into a given cell type. Expanding on the ooplasm extrusion experiments of Driesch and Morgan, Crampton (1896) provided persuasive evidence for the preferential distribution of determinative cytoplasmic materials to one daughter cell. In the first division of the gastropod, *Ilyanassa*, a sac of cytoplasm, termed the "polar lobe" is transiently segregated and then distributed to one daughter. When this lobe was mechanically removed, Crampton found the embryo to undergo normal cleavage, but to give rise to a larva lacking mesodermal derivatives. This demonstrated that mesodermal determinants are segregated to the polar lobe, and, more importantly, that the identity of specific blastomeres could rely on character of the cytoplasm it receives upon cleavage. These results were confirmed and elaborated upon by Wilson in the mollusc *Dentalium* (1903, 1904). Roux's proposal of Qualitative Division was thus shown to be correct when applied to the cytoplasm instead of the nucleus.

Boveri had proven that sea urchin embryos also contain a stratified cytoplasm (1902). Finally, it was Wilson who explained that the conflict between Roux's results with frogs and Driesch's results with sea urchins rests in the distinctive ways that the two organisms distribute these components during their initial cleavages:

> "...in the sea-urchin egg, the first two cleavages cut the three strata of the egg symmetrically; both, therefore, are purely quantitative. The first two or four cells thus receive identical portions of each of the three zones, and are ...equipotential and totipotent. In the mollusc or annelid, on the other hand, at the first cleavage the lower stratum passes bodily into one of the two cells. This division, therefore, is qualitative, allotting different combinations of ooplasmic materials to the first two blastomeres; hence the mosaic like character of cleavage from the beginning (Wilson, 1925).

Wilson further points out that the third cleavage of the sea urchin embryo is mosaic, producing unequal cells. While explaining that "regulative" eggs can undergo mosaic divisions, Wilson emphasizes that distribution of cytoplasm alone cannot account for the complete development of an organism. That is, regulative (or inductive) interactions direct development once cell division has distributed necessary determinants. This culmination of fifty years of classical embryology set in place the importance of intrinsically asymmetric divisions to development.

Asymmetrically distributed determinants

Wilson's interpretations of the classical data have been substantiated by modern experimentation, which has led to the identification of asymmetrically localized RNAs and proteins within oocytes, as well as to the characterization of factors involved in inductive processes. The best understood example of localized determinants instructing pattern formation is offered in the case of *Drosophila*. It should be noted that early embryonic divisions in *Drosophila* technically do not qualify as asymmetric, since early nuclear divisions occur without cytokinesis. Nonetheless, much can be learned from the *Drosophila* model for establishing the anteroposterior [A-P] axis. A-P axis formation relies on the anterior localization of maternal bicoid RNA and the posterior localization of maternal nanos RNA during oogenesis (Berleth et al., 1988; Wang and

Lehmann, 1991). Upon translation, the bicoid and nanos products diffuse and form complementary gradients along the anteroposterior axis. High levels of bicoid, a transcription factor, activate expression of another transcription factor, hunchback, in the anterior nuclei (Driever and Nüsslein-Volhard, 1989; Struhl et al., 1989), while nanos, an RNA binding protein, negatively regulates hb translation in the posterior (Tautz and Pfeifle, 1989; Wharton and Struhl, 1991). The resultant pattern of hb expression activates a cascade of gene expression that specifies pattern along this axis (Struhl et al., 1992).

Pattern development in the vertebrate, *Xenopus*, may differ greatly from *Drosophila*, but some parallels can be drawn. In the oocyte, a number of different RNAs have been found to localize to the animal or vegetal pole (Ding and Lipshitz, 1993; Klein and Melton, 1994). Three RNAs found specifically in the vegetal pole, Veg-1 (Melton, 1987; Rebagliati et al., 1985), TGFß-5 (Kondiah et al., 1990; Schwartz et al., 1992), and Xwnt-11 (Christian et al., 1991; Ku and Melton, 1993) appear to function in the induction or patterning of the mesodermal germ layer (Klein and Melton, 1994; Ku and Melton, 1993; Thomsen and Melton, 1993). One vegetally localized RNA, Xcat-2, encodes a homolog of nanos (Mosquera et al., 1993), suggesting that Xcat-2 may repress translation of important regulatory genes in the vegetal hemisphere.

Although localized RNAs have not yet been identified in *C.elegans* oocytes, several lines of evidence suggest that localized determinants do exist. For example, intestinal cells derive exclusively from P_1, the posterior daughter of the first division (AB is the anterior daughter). Blastomere isolation experiments and cytoplasmic mixing experiments show that P_1 specifically acquires intestinal determinants which may be present but inactive before the first division (Edgar and McGhee, 1986; Schierenberg, 1985). A promising route to identifying a localized determinant may be through the glp-1 RNA. glp-1 encodes a

transmembrane protein of the lin-12 and Notch family (Yochem and Greenwald, 1989) that is involved in numerous inductive interactions during *C.elegans* development, including induction of anterior blastomere fates by posterior blastomeres (Austin and Kimble, 1987; Mello et al., 1994; Priess et al., 1987). Translation of the uniformly distributed maternal glp-1 mRNA is restricted to the descendants of the AB cell. The mechanism of this regulation includes a 3' UTR sequence which has similarity to the nanos response element and mediates translational repression in the cells derived from P_1 (Evans et al., 1994; Wharton and Struhl, 1991). Again reminiscent of nanos regulation of hunchback, these results argue for the presence of a nanos-like RNA-binding protein localized to the P_1 cell. Such a localized factor could also be involved in the asymmetric expression of another gene, *skn-1*, which turns on in the P_1 cell daughters and is required for pharyngeal cell fate (Bowerman et al., 1993; Mello et al., 1992)

A possible vehicle for a posteriorly localized determinant is provided in the P granules, which attach to the posterior cortex and segregate to P_1 in the first division (Strome and Wood, 1982; Strome and Wood, 1983). Perhaps most intriguing, however, are the five *par* genes. Mutants in these genes fail to partition the P granules and disrupt key events in cytoplasmic reorganization within the embryo (Kemphues, 1988; Kirby, 1990; D. Shakes, D. Morton, and K. Kemphues, unpublished). Although little is known about the mechanism of action of these genes (only *par-2* has been cloned; it encodes a putative transcription factor with an ATP binding domain and a zinc finger motif (Levitan et al., 1994)), they promise to enlighten our understanding of how asymmetry is established in this first division of the *C.elegans* embryo.

These selected examples illustrate how the mechanism of asymmetric localization of determinants is widely used to specify fates in early stages of embryonic development. It is possible that oocytes and embryos, in contrast to

cells later in development, adopt this strategy in response to the unique challenges that confront them: First, the initial divisions of the embryo must establish and coordinate the axes of the animal as well as its germ layers. Second, the egg, once laid, receives no positional information from surrounding cells. Thus, the polarity signals it encounters are limited to its interactions with neighboring cells during oogenesis and, upon fertilization, the point of sperm entry. *Drosophila* and *Xenopus* eggs meet these challenges by dedicating time and resources to the creation of a polarized egg cell. Fertilization, in these cases, triggers a series of rapid divisions which are fueled by the products of maternally provided RNAs. For *C.elegans*, the investment in the oocyte may be less pronounced, as no clear asymmetries have been recognized in the unfertilized egg. Fertilization itself appears to activate a microfilament dependent reorganization of the cell into the requisite polarized form (Hill and Strome, 1988; Hill and Strome, 1990). The localization of determinants and their differential distribution during early embryonic divisions are, thus, an effective approach to solving the multi-dimensional problems that face the embryo. But is this mechanism of fate specification utilized in later divisions?

After the initial embryonic divisions, cells enjoy a wealth of positional information offered by surrounding cells. Still, there is formally no reason that the mechanism of asymmetric localization of determinants should not be reiterated in these cells. Indeed, positional cues supplied by surrounding cells may cause the localization of a substance within a dividing cell, resulting in the birth of two intrinsically different daughters. This would constitute an environment-dependent intrinsic asymmetry. Alternatively, later cells could generate polarity independent of their environment and give rise to two distinct daughter cells. This ability to produce distinct daughters irrespective of the surroundings has been observed in two prokaryotes, *Caulobacter crescentus* and *Bacillus subtilis* (Shapiro, 1993a),

as well as for directional growth of *S. cerevisiae* (Chant and Pringle, 1991). These organisms present illuminating examples of how polarity can be created within a single cell. As yet, however, neither of these approaches to generating an intrinsically asymmetric division has been demonstrated in multicellular organisms beyond the early embryonic cleavages.

Several genes involved in late asymmetric fate decisions have been identified in *C.elegans*, through examining cell lineages in mutant animals (Horvitz and Sulston, 1980; Sulston and Horvitz, 1977). This approach relies on the rationale that loss of function of an asymmetrically required determinant will cause both daughter cells to adopt the fate of the cell which normally does not need the factor. Theoretically, a subset of these genes could encode fate determinants that are either specifically partitioned to one daughter cell or are required for such partitioning. To date, this method has identified genes that are specifically expressed in one daughter cell, but so far no factor has been found which is itself distributed non-uniformly in an asymmetric division (Bowerman et al., 1993; Evans et al., 1994; Way et al., 1992). One promising mutant, *lin-17*, perturbs asymmetry in multiple divisions, leading to speculation that the wild-type gene product may be generally needed in asymmetric divisions (Sternberg and Horvitz, 1988). Further cloning and characterization of the *lin-17* gene product have not been reported.

In *Drosophila*, two mutants that appear to disrupt asymmetric divisions were identified in screens for genes affecting the PNS (see review, Jan and Jan, 1990b). My work centers on one of these genes, numb, which seems to affect asymmetric divisions in both external sensory organ [es] and chordotonal organ [ch] development (Uemura et al., 1989). I have focused on numb action in external sensory organs primarily because es organ development is better understood than ch development. While the differentiation of es organ cells has

always posed the interesting question of how fates are specified, the potential for understanding the generation of intrinsic asymmetry through this system adds a new dimension to the fate issue. Before I elaborate on our current understanding of the processes that direct the fate of the four cells, I will provide some background on the external sensory organ system.

Development of external sensory organs

General Background

There are four types of sensory neurons which innervate the different sense organs of the *Drosophila* peripheral nervous system: External sensory neurons innervate structures which lie at the cuticle surface to receive mechanical and chemical stimuli from the environment; chordotonal neurons associate with subepidermal organs that detect stretching and vibration; multiple dendritic neurons consist of bipolar neurons, tracheal neurons, and neurons which have extensive dendritic arborizations beneath the epidermis (Hartenstein, 1988; McIver, 1985; Zacharuk, 1985); and photoreceptors cells in the compound eye absorb and transduce light (Smith et al., 1991; Tomlinson, 1988). My work concentrates chiefly on the neurons and accessory cells which comprise the external sensory [es] organ.

The prototype es organ is represented as a four-cell structure with a trichogen, tormogen, neuron and thecogen cell (Figure 1A). The tormogen and trichogen form the stimulus receiving structure, with the tormogen forming a socket and the trichogen elaborating either a hair (for trichoid sensilla) or a domed papilla (for campaniform sensilla). These outer support cells may be innervated by a single peripheral neuron and its thecogen (sheath) cell, or by multiple neurons in the case of a more complex organ. The four principal cells are derived from two rounds of division of a Sensory Organ Precursor cell [SOP] (Bate, 1978; Bodmer et al., 1989; Hartenstein and Posakony, 1989)(Figure 1B). Analysis of es organ formation in the adult notum indicates that the first division lies in the plane of the epidermis and yields two secondary precursors, termed IIa and IIb (Hartenstein and Posakony, 1989). This SOP cell division constitutes the first of three asymmetric divisions in this lineage. The secondary precursors are visually identical but display a difference as they arrest for different lengths of

time before entering the second round of division. The IIa cell divides first , and the IIb cell divides 1-2 hours later, together giving rise to four cells in the epidermal layer which are morphologically indistinguishable (without appropriate markers) from each other and neighboring epidermal cells. The introduction of BrDU at different time points during es organ development indicates that the IIa cell gives rise to the trichogen and tormogen, while the IIb cell descendants form the neuron and thecogen (Bodmer et al., 1989; Hartenstein and Posakony, 1989). In the case of poly-innervated es organs, the additional neurons are thought to arise from further divisions of the neuron cell (Bodmer et al., 1989). This lineage is reminiscent of lineages described for external sensillae of other insects (Bate, 1978).

The neuron is the first cell to become visibly distinct: It enlarges and delaminates from the epidermal layer. Subsequently, the apical regions of the three support cells converge as their cell bodies shift to a subepidermal position. During morphogenesis, the trichogen and tormogen cells form concentric sheaths around the thecogen cell near the cuticle surface, and the neuron extends its dendrite apically, such that the dendrite burrows into the overlying, distended portion of the sheath cell. Cytodifferentiation ensues as the trichogen and tormogen extend hair and socket structures, respectively. Simultaneously, the outer cells become polyploid through one (tormogen) or two (trichogen) rounds of endoreplication, which ostensibly leads to their enlarged cell volume. The neuron also grows an axon, which fasciculates with axons of neighboring organs and ultimately grows out to the thoraco-abdominal ganglion (Hartenstein and Posakony, 1989). The organ also associates with a fifth cell, a glial cell of unknown origin, which is not lineally related to the other es organ cells (Bodmer et al., 1989; Hartenstein and Posakony, 1989).

Since the focus of this thesis is the relevance of asymmetric divisions to the differentiation of the four es organ cells, I will present an extensive review of genes affecting proper fate specification in these cells. For context, however, I will first briefly summarize the current understanding of the processes of selecting the SOP and determining organ type and subtype. Several thorough reviews discuss these topics in depth (Artavanis-Tsakonas and Simpson, 1991; Ghysen and Dambly-Chaudière, 1993b; Ghysen et al., 1993a; Giangrande and Palka, 1990; Jan and Jan, 1994b).

Selection of the SOP

The Sensory Organ Precursor is selected from a field of epidermal cells in a manner similar to the selection of neuroblasts in the CNS. This selection can be subdivided into two phases: First, a group of adjacent epidermal cells expresses the Achaete Scute Complex [AS-C] genes. This "proneural cluster" is thus endowed with the potential to differentiate as SOPs (Campuzano and Modolell, 1992; Cubas et al., 1991; Ghysen and Dambly-Chaudiere, 1988a). Competition between cells in this cluster constitutes the second phase, where cells signal their neighbors to abandon the SOP fate and become epidermal. This process of "mutual inhibition" occurs via the neurogenic genes, resulting in the emergence of one victorious cell which up-regulates its levels of AS-C genes to become the SOP cell, while causing its neighbors to decrease their expression of this set of genes (Ghysen and O'Kane, 1989). The AS-C genes encode basic helix-loop-helix proteins which can dimerize with the ubiquitous daughterless protein and activate transcription from responsive promoters (Cabrera and Alonso, 1991; Caudy et al., 1988; Jarman et al., 1993b). This activation, directly or indirectly, leads to the expression of the "neural precursor genes" which are hypothesized to impart neuronal characteristics to these cells (Bier et al., 1992; Brand et al., 1993a; Vaessin et al., 1991).

Type and subtype determination

In the course of its development, the es organ faces multiple fate decisions. Three of these choices appear to be resolved through the expression of a transcription factor which favors one of two fates: *cut*, a homeobox gene, causes bipotential precursor cells to adopt es rather than ch organ fate (Blochlinger et al., 1991; Bodmer et al., 1987b); *pox-neuro*, which contains a paired-box domain, determines that the es organ be poly-innervated instead of singly innervated (Dambly-Chaudiere et al., 1992; Nottebohm et al., 1992); and the homeobox-containing *BarH1* and *BarH2* genes confer the subtype of trichoid sensillum over the campaniform identity onto these organs (Higashijima et al., 1992). While mutations in any of these genes alter the type or subtype of organ, the neuron and accessory cells differentiate appropriately in these mutants, confirming that these genes are not required for the correct specification of the four individual cell fates.

Differentiation of the four cells

The central subject of this dissertation is whether any of the three divisions giving rise to an es organ are intrinsically asymmetric. If so, what constitutes the difference between sister cells, and how is this difference created? I approached this question through a mutation in the *numb* gene, which was hypothesized to affect the division of the SOP cell, rendering the division symmetric (Uemura et al., 1989). I have pursued this hypothesis and have determined that numb plays a critical part in generating intrinsic asymmetry in the division of the SOP cell, as well as in the subsequent divisions. To understand the relevance of this asymmetry and to appreciate how other genes may contribute to the establishment or readout of this asymmetry, it is useful to review current literature on the genes required for es cell fate specification.

Within the last five years, the repertoire of genes demonstrated to act in es cell fate specification has vastly expanded. Although the advances have yet to coalesce into a thorough understanding of the process, the information so far unveiled offers a tantalizing glimpse of the diverse mechanisms which may direct these cell fates. I will review the current literature that demonstrates the roles of various genes in es cell differentiation and interject some new interpretations of existing data. Three themes emerge from the current data: First, there are surprisingly many genes which, when mutated, perturb the intrinsically asymmetric divisions; second, specification of the four cell fates appears to be a stepwise process, consisting of fate decisions at three asymmetric divisions; third, though these three divisions yield different cell types, they may utilize the same set of genes to generate asymmetry. Thus, genes required to confer IIb versus IIa cell fate seem to be required again for hair cell rather than socket, and neuron fate as opposed to sheath. Conversely, genes necessary for IIa fate may also be needed to form the socket and sheath cells. Table 1 lists the genes that I will discuss and their described effects on each asymmetric fate decision.

Cell-cell communication via neurogenic genes

Key evidence for the role of cell-cell communication during these asymmetric divisions comes from the findings that *Notch [N]* and *Delta [Dl]* are required for asymmetry of all three divisions. These results indicate that any intrinsic asymmetry mechanism involving numb must also account for signaling between N and Dl. Originally identified as members of the neurogenic class of mutants which cause hypertrophy of the embryonic neurectoderm (Lehmann et al., 1981), *Notch* and *Delta* encode transmembrane receptors with multiple EGF repeats (Kidd et al., 1986; Kopczynski et al., 1988; Vaessin et al., 1987; Wharton et al., 1985). They are thought to be receptor (N) and ligand (Dl) in a signaling pathway wherein cells inhibit neighbors from acquiring a particular fate

(Artavanis-Tsakonas, 1988; Fehon et al., 1990; Xu et al., 1990). N and Dl proteins are expressed in the same cells at roughly the same time during CNS development, and activation of signaling is thought to occur through a post-translational mechanism (Kooh et al., 1993). The need for these neurogenic genes in es cell fate determination echoes previous findings that this group of genes is utilized by the organism in multiple cell fate decisions during development, from the selection of mesodermal cells (Corbin et al., 1991) to the restriction of the number of specialized follicle cells during oogenesis (Ruohola et al., 1991).

The effects of *Notch* mutation on es cell fate determination were originally undetected due to the requirement for *Notch* in the lateral inhibition step of SOP cell selection. Hypomorphic alleles or flies heterozygous for a strong loss of function allele display hypertrophy of SOP cells, as evidenced by supernumerary bristles, but these SOPs apparently differentiate normally. The first report of an alternative bristle phenotype in *Notch* mutants describes the analysis of a temperature sensitive allele. Although some shifts to non-permissive temperature reflect the role of *Notch* in restricting the number of SOP cells, forming extra microchaetae, shifts of slightly older animals result in apparent loss of microchaetae (Shellenbarger and Mohler, 1978). On further analysis, Hartenstein and Posakony (1990) determined that the bald patches on the cuticle actually reflect the transformation of the hair and socket cells to neurons.

The subsequent analysis of a temperature sensitive allele of *Delta* yielded comparable results (Parks and Muskovitch, 1993). If pupae are placed at the non-permissive temperature during the period preceding SOP division, tufts of extra bristles form, illustrating the role of *Delta* in selecting a single precursor. Pupae which are subjected to temperature shifts after the division of the SOP develop mutant bristles in which the outer support cells have been transformed

into inner cells. Two classes of transformations are commonly observed: Either the hair and socket are transformed into an additional neuron and sheath, producing two sets of neuron and sheath pairs, or all four cells beneath the bald cuticle have the appearance and immunogenic profile of the neuron. These data support the model that fate specification is a sequential process, where the secondary precursors must first acquire their proper identities as the hair/socket precursor (IIa) or the neuron/sheath precursor (IIb) before the final four cells can develop properly. The *Notch* and *Delta* phenotypes can be explained as a failure to specify the fate of the IIa cell, resulting in the formation of two IIb cells and, ultimately two pairs of inner cells. This effect is opposite to the transformation observed in *numb* mutants. The appearance of four neurons under some N^- or Dl^- bald patches invokes the additional need for *Notch* and *Delta* in the neuron versus sheath fate decision. Thus, when Notch or Delta function is removed during both the secondary precursor fate decision and the neuron/sheath fate decision, the two neuron/sheath precursors formed divide and produce four cells which all differentiate as neurons.

A third neurogenic gene, *neuralized*, also appears to contribute to the es cell fate. Mosaic animals containing large neu^- patches are unable to form outer support cell structures, despite forming vast arrays of neurons underneath the bald cuticle. When the recombination is induced later, to form small mosaic patches within a single organ, a new phenotype is observed: The hair cell is duplicated at the expense of the socket cell (G. Feger, in preparation). This twin hair phenotype suggests that neurogenic genes are also involved in the hair/socket fate decision, a role which was not revealed by the ts alleles (This is understandable, since shifts which may have affected this decision invariably also affect the secondary precursor fate, yielding two neuron/sheath precursors

and masking the need for the neurogenic genes in the hair/socket decision altogether).

Investigation of *big brain [bib]* reveals that this mild neurogenic gene is not involved in es organ cell fate determination (Dietrich and Campos-Ortega, 1984; Rao et al., 1992), despite its role in SOP selection (Goriely et al., 1991). This result is consistent with other findings that *bib* does not interact genetically with the other neurogenic genes (de la Concha et al., 1988).

The effects of the remaining neurogenic genes, *mastermind* and *Enhancer of split*, have yet to be reported, but one would predict that they, like *N*, *Dl*, and *neu*, will act in all three asymmetric divisions. Extrapolating from their roles in neurogenesis, one could postulate that this group of genes may transduce an inhibitory signal from the IIb/hair/neuron to dissuade the neighboring (sister) cell from taking the same fate.

Nuclear proteins

In addition to the neurogenic gene, *neuralized*, several genes involved in sensillum cell differentiation have been identified whose products are anticipated to localize to the nucleus. These gene products share no obvious functional relationships, but they all appear to affect multiple asymmetric cell fates. Among the three proteins to be discussed, *Hairless* alone lacks compelling homology to previously described factors. It is loosely placed in the "nuclear protein" category based on its high proportion of basic residues, which may mediate binding to DNA (Bang and Posakony, 1992; Maier et al., 1992). The missing bristles and double sockets of *Hairless* flies were originally described by Bridges and Morgan in 1923. After more thorough investigation, Lees and Waddington (1942) proposed that the double sockets reflect the transformation of the hair cell to an additional socket. This hypothesis was confirmed through analysis of antibody markers, BrDU incorporation patterns, and bristle ultrastructure (Bang et al.,

1991). As for the Hairless bald phenotype, which is more prominent in stronger alleles, examination with cell specific markers indicate the SOP is never formed. Thus, unlike the bald phenotype induced by shifting N^{ts} or D^{ts} to non-permissive temperature, the *Hairless* bald cuticle results from the failure to form the SOP and all its descendants (Bang et al., 1991). Although this role precedes the need for Hairless in hair cell fate, understanding *Hairless* in this context may be instructive to deciphering its role later, during es cell fate specification. The bald phenotype reiterates the previously described capacity of Hairless to antagonize lateral inhibition by neurogenic genes (Vaessin et al., 1985).

As microchaetae SOP cells divide, elevated levels of Hairless RNA appear in what may be secondary precursor cells and in tormogen and trichogen cells shortly after they become visually distinguishable (Bang and Posakony, 1992). Protein localization data have not been reported for Hairless.

In a model recently presented by Posakony (Posakony, 1994), H and Su(H) are proposed to act in the secondary precursor fate decision and neuron/sheath decision in addition to their established roles in the hair/socket decision. Since strong alleles of *Hairless* fail to form SOPs, proof of a role for *Hairless* in the secondary precursor fate decision would require the induction of H^- clones via a recombination event that occurs during the division of the SOP. This non-trivial experiment should nonetheless be possible given the frequency of mitotic recombination using the Flp/FRT method (Golic and Lindquist, 1989), assuming that perdurance is not a factor. A suggestion that *Hairless* can act in the IIa/IIb decision already exists from experiments where Hairless expression from a heat shock promoter is induced at various times during microchaetae formation (Bang and Posakony, 1992). Induction early, during SOP selection, leads to supernumerary SOP cells, confirming the action of *Hairless* to promote SOP formation. hs-H expression later in microchaetae development yields the

socket to hair transformation predicted by its requirement in the hair/shaft fate decision. Induction of hs-Hairless at an intermediate time, however, causes a missing microchaetae phenotype. This phenotype has been attributed to the action of Hairless in antagonizing the role of Notch in selecting a single sensory neuron from the four progeny of the SOP (Bang and Posakony, 1992). Given the current model that the determination of the neuron is a stepwise process, the missing microchaetae phenotype is consistent with *Hairless* action in the first step: specification of the IIb cell.

Loss of function alleles of *Suppressor of Hairless* act dominantly to suppress both the bristle loss and double socket Hairless phenotypes. Duplications or gain of function alleles of this locus conversely enhance the *Hairless* phenotypes (Ashburner, 1982; Lindsley and Zimm, 1992; Nash, 1965; Nash, 1970). By this and other criteria, *Su(H)* acts as a neurogenic gene in the generation of adult PNS (Schweisguth and Posakony, 1992). Since homozygous *Su(H)* causes lethality in early pupae, the effects of removing *Su(H)* in the developing bristle were examined by generating *Su(H)* somatic clones in an otherwise heterozygous animal. When clones are induced late in es cell differentiation, the phenotype predictably reflects the reverse of the *Hairless* double socket phenotype: An apparent socket to hair transformation yields double hairs with no sockets (Schweisguth and Posakony, 1994b).

In light of the stepwise model for es organ cell differentiation, re-evaluation of previously confounding data lends compelling support to the suggestion that *Su(H)* acts in the IIa/IIb fate decision. While small mosaic clones of *Su(H)* give rise to bristles with double hairs and no sockets, large *Su(H)* clones curiously give rise to bald patches (Schweisguth and Posakony, 1994b). Based on the proposed actions of *Su(H)* in SOP selection and hair/socket fates alone, one would predict large Su(H) clones to produce supernumerary SOP cells which

each form two shafts, a normal neuron and a sheath cell. Externally, these patches should appear as tufts of double hair-containing bristles. The naked cuticle which is observed implicates another role for *Su(H)*. If *Su(H)* acts, as the neurogenics do, in the secondary precursor fate decision, all of the SOP cells in a mutant patch would divide to form two neuron/sheath precursors. Lacking socket/hair precursors, the external structures would not form, and the cuticle would be bare, consistent with the observation. This hypothesis could be tested by examining the tissue beneath the bald patches for extra neurons. If *Su(H)* acts to ensure asymmetric fates between the secondary precursors, ectopic expression of Su(H) at the time when secondary precursors emerge should induce formation of two IIa cells. Although a heat shock-Su(H) construct has been made and tested, the four outer cell phenotype that would be expected if two IIa cells were formed was not observed (Schweisguth and Posakony, 1994b). I argue that, considering the unusually long heat shock regimen required to induce adequate levels of Su(H) from this construct (3 X 60 min at 37°C, separated by two 90 min intervals at 25°C), induction of Su(H) in the 16 hour pupae may not have been sufficient to affect the secondary cell fate decision which occurs at 16-18 hours after puparium formation. Thus, the hypothesis that Su(H) affects the secondary precursors' fate decision remains to be adequately tested.

Su(H) encodes a protein with 75% homology to the murine J_K Recombination signal Binding Protein [RBP] (Furukawa et al., 1992; Schweisguth and Posakony, 1992). The binding sites for the mouse site specific DNA binding protein are essential and sufficient for V.DJ recombination (Akira et al., 1987; Hesse et al., 1989; Kawaichi et al., 1991). Sequences within the protein reveal a catalytic domain common to the integrase class of recombinases (Matsunami et al., 1989). Although recombinase activity has not been demonstrated with the

Drosophila protein, this homolog has been shown to bind DNA with a similar sequence specificity to the murine J_K RBP (Hamaguchi et al., 1989b). During microchaetae development, the expression of Su(H) RNA has been detected in the tormogen and trichogen cells (Schweisguth and Posakony, 1992).

A recent study of Su(H) protein in tissue culture cells suggests that Su(H) is anchored to the plasma membrane via interaction with Notch. Activation of N by Dl in this in vitro system releases Su(H), enabling its translocation to the nucleus (Fortini and Artavanis-Tsakonas, 1994). These investigators also report the ability of Su(H) protein to interact directly with Notch protein in a two hybrid system. Although these interactions remain to be proven in vivo, the observations present an interesting integrated model by which neurogenic genes and Su(H), may initiate the IIa, socket and sheath fates.

tramtrack [ttk] is another nuclear protein that is required for proper es cell fate determination. It is allelic to the previously isolated mutant, *oversensitive* (Jan and Jan, 1990b); (Guo et al., submitted), which transforms all four cells to neurons (Salzberg et al., 1994). Ectopic induction of ttk from a heat shock promoter, as well as studies of mosaic *ttk* patches on the adult notum, suggests that *ttk* may be required in all three asymmetric fate decisions in the PNS, acting in the same direction as the neurogenic genes (Guo et al, submitted). The *ttk* locus encodes two zinc finger proteins, p69 and p88, which have been shown to repress transcription of a number of genes involved in segmentation (Brown and Wu, 1993; Harrison and Travers, 1990).

Insight into the pathway leading to ttk activation may be gained through the understanding of ttk's role in the selection of the R7 photoreceptor cell. Selection of this cell occurs through activation of the sevenless receptor tyrosine kinase via binding by the ligand bride-of-sevenless which resides on the neighboring R8 cell (Greenwald and Rubin, 1992; Zipursky et al., 1992). The

response to this activation is mediated through a ras-dependent signaling pathway (Hafen et al., 1993; Simon et al., 1992). In flies lacking the p88 protein, a number of outer photoreceptor cells appear to assume the R7 cell fate. Thus, p88 may be required to repress genes which activate the R7 fate in the non-R7 photoreceptor cells (Xiong and Montell, 1993). A candidate for one such activating gene *is seven-in-absentia* [sina], which encodes a zinc finger protein required for R7 cell fate (Carthew and Rubin, 1990; Moses, 1991). A bristle defect for *sina* has recently been observed which is opposite to the ttk transformation . If sina and ttk antagonize each other to specify R7 fate, a similar competition may take place in the es organ cells to specify IIb fate. Protein expression data indicate that ttk is expressed in the three non-neuronal cells (Guo et al., submitted), suggesting that ttk may act later to repress neuron specific genes.

Other interesting factors

A number of other genes have been identified that affect es cell specification. Although they contain interesting homologies, it would be premature at this time to speculate on their relationships to the other described factors. One of these genes is *musashi [msi]*, mutants of which form additional hair and socket cells at the expense of the neuron and sheath. The relatively frequent occurrence of a range of hair and socket phenotypes, from two hairs and a single socket to two sockets and a single hair, suggests that musashi is not strictly comparable to the other mutants. Whereas mutants of *N*, *Dl*, *Su(H)*, *H*, *ttk*, *tws* or *nb* overwhelmingly favor one fate over another in all three asymmetric divisions, the *msi* null allele consistently biases only the secondary precursor fate decision, forming extra outer cells which may differentiate as hair or socket. The musashi protein contains two regions that strongly resemble RNA binding domains and shows greatest homology to two putative RNA binding proteins of

Xenopus (Nakamura et al., 1994). Based on this homology and the nuclear localization of the protein, Nakamura et al. propose that musashi is involved in RNA splicing. A P[lacZ] enhancer trap construct inserted in the musashi 5' regulatory region expresses the reporter gene in all four cells of the developing es organ (Nakamura et al., 1994).

The fate of the es organ cells is also dependent on a gene which appears to be involved in cell division and morphology. The gene, *twins [tws]*, encodes the regulatory subunit, B, of serine/threonine protein phosphatase type 2A (Uemura et al., 1993). A hypomorphic mutation in *tws* displays a specific defect in the IIa vs. IIb cell fate, causing the differentiation of two hairs and two sockets (Shiomi et al., 1994). Somatic clones that remove the strongest existing allele of *tws*, *twsP*, display the same phenotype, leaving the hair/socket fate decision intact. Analysis of *cdc55*, the *tws* homolog in *S. cerevisiaie*, suggests a role in yeast bud formation. The elongated buds created in *cdc55* cold-sensitive mutants are suppressed in the *BEM-2* mutant, which is defective for a gene required for bud emergence (Healy et al., 1991).

key questions

The genes described in this review constitute formidable support for the model that the three asymmetric divisions which generate es organ cells share a common mechanism to choose between two fates. Based on the functions of these proteins in other developmental decisions, it appears that interaction between cells, specifically lateral inhibition, dictates one aspect of establishing or maintaining asymmetric cell fates. Despite the extensive similarities among the three divisions, it should be noted that some genes are expected to be unique to particular divisions, since the terminal fates of the four cells are distinct. A mutation in the gene *sanpodo* has recently been described to affect the neuron/sheath decision specifically (Salzberg et al., 1994). Although analysis of

null alleles at this locus is necessary to confirm this specificity, such a result would exemplify a point where mechanisms diverge. Similarly, *tws* and *msi* may be genes that are specific to the IIa/IIb fate decision.

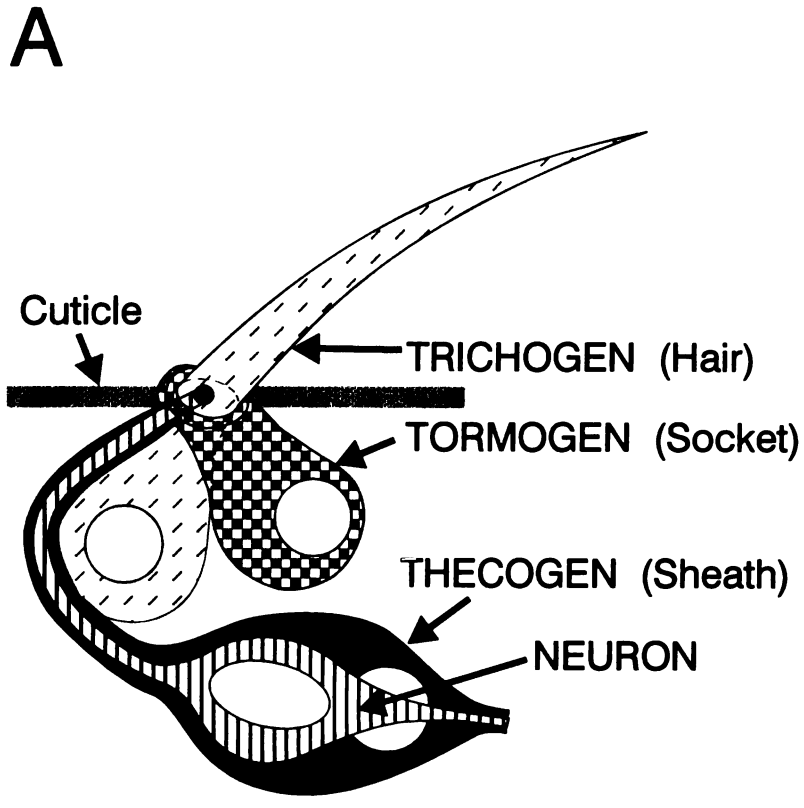
In previously characterized cases where the "cassette" of conserved genes acts, the cells involved are not directly related by lineage (Campos-Ortega, 1988a; Corbin et al., 1991; Ruohola et al., 1991). Considering the stereotype lineage of the es organ cells, I ventured to ask whether lineage, or inherited differences, contribute to these asymmetric decisions. That is, are the two cells distinct at birth, or are they interchangeable, with differences arising through interactions with the environment? This point is not trivial. While the lineage relationship between the es organ cells offers the potential for involvement of inherited factors in determination, I emphasize that the lineage pattern alone does not necessarily ensure the existence of intrinsic differences. This point is vividly illustrated by the invariant lineage observed for the cells of *C. elegans*. Although the worm's strict adherence to the lineage pattern prompted many to believe that cell fates are predetermined, or autonomous (Sternberg and Horvitz, 1984), ablation and genetic analyses proved that cell interactions can mediate cell fate decisions and still produce an invariant lineage (Ferguson et al., 1987; Sternberg and Horvitz, 1989).

Shortly after I began this project in the summer of 1990, the detailed characterization of the N^{ts} allele, describing a dual role in SOP selection and single sensory neuron selection, was published (Hartenstein and Posakony, 1990). The interpretation of thiese data at the time was that the fates of the sensillum cells were not fixed at the four-cell stage, but that identities were acquired via cell-cell interactions. Es cell fate determination was not regarded as a stepwise process consisting of successive asymmetric divisions, and the importance of lineage was in doubt. Still, the unusual phenotype of the *numb*

mutant provided hope that the asymmetric divisions in the es lineage were important to confer fate. I was interested in how these four distinct fates were specified. Ideally, I hoped that *numb* would lead me to understand how asymmetries are established in terminal divisions, or more simply put, how two sister cells acquire different fates. My first task was to determine whether the *numb* mutant phenotype represents a transformation at the level of the secondary precursor cells, which would confirm that the mutant perturbs an asymmetric division. In Chapter 1, I describe the experiments that firmly establish *numb*'s action on asymmetry of the SOP division. I provide evidence that numb is also required in the division of the IIa cell. Most importantly, I show that numb protein itself is the asymmetrically distributed factor which generates a difference between the IIa and IIb cells. I examine the effect of twins mutation on numb localization in Chapter 2. In Chapter 3, I present a model for how numb, the cell intrinsic determinant, may impinge on inter-cellular signaling mediated through Notch. Finally, I provide a prospectus in chapter 5, elaborating on the next questions to be pursued.

Figure 1 Diagrams of es organ and cell lineages

(A) Schematic drawing of a simple es organ. The outer hair and socket structures are formed, respectively, by the trichogen and tormogen cells. These support cells are innervated by a neuron whose dendrite is ensheathed by the thecogen, or sheath cell (trichogen and tormogen form subsequent layers of sheath around tip of the dendrite.) The four cells are related by the division pattern depicted in (B). The SOP cell (also referred to as SMC and pI) divides forming the secondary precursors IIa and IIb. IIa next divides producing cells that eventually form the hair and socket. IIb divides shortly after IIa, giving rise to cells that differentiate as the neuron and sheath.


A
Cuticle
TRICHOGEN (Hair)
TORMOGEN (Socket)
THECOGEN (Sheath)
NEURON

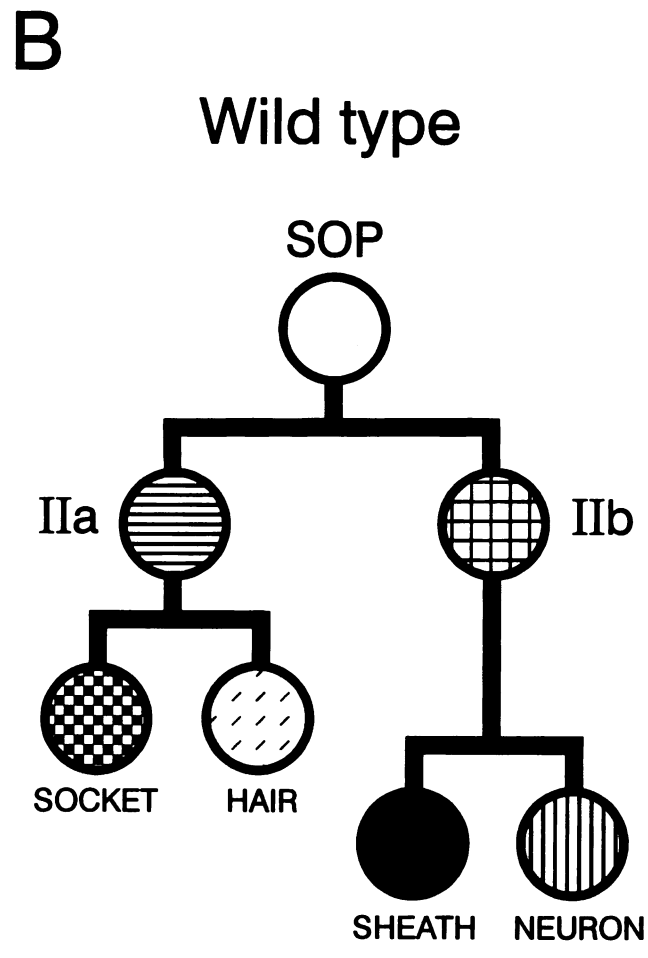

B
Wild type
SOP
IIa
IIb
SOCKET
HAIR
SHEATH
NEURON

Figure 1

Table 1. Genes involved in es organ differentiation

The first column lists genes which are discussed in the text. Loss-of-function phenotypes are catalogued in the subsequent columns. While "extra SOPs" refers to the multiple SOPs that form in the relevant mutants, the use of "extra" in the other columns refers to the fate transformation that causes both sister cells to differentiate identically, rendering an "extra" cell of one cell type at the expense of the other cell type. Phenotypes which are predicted based on the transformation observed in a gain-of-function or overexpression strain are shown in italics. Question marks indicate mutant phenotypes which have not yet been adequately examined.

TABLE 1 Mutants That Affect es Organ Differentiation

MUTANT	PHENOTYPES IN DIFFERENT FATE DECISIONS			
	epidermal vs. SOP	IIa vs. IIb	socket vs. hair	sheath vs. neuron
Notch	extra SOPs	extra IIb	*extra hair*	extra neuron
Delta	extra SOPs	extra IIb	?	extra neuron
neuralized	extra SOPs	?	extra hair	extra neuron
Su(H)	extra SOPs	?	extra hair	?
Hairless	no SOP	*extra IIa*	extra socket	?
tramtrack	no effect	extra IIb	*extra hair*	extra neuron
sina	no effect	extra IIa	?	?
twins	no effect	extra IIa	?	?
musashi	no effect	extra IIa	extra hairs and extra sockets	?

CHAPTER 1

Asymmetric distribution of numb protein during division of the Sensory Organ Precursor cell confers distinct fates to daughter cells

INTRODUCTION

A central question in the study of development is how a single cell can give rise to two cells that have distinct fates. Any such division in which the daughter cells ultimately have different identities has been defined as an asymmetric division (see Horvitz and Herskowitz, 1992 for an extensive review). The asymmetry may be the result of the unequal distribution of determinant(s) to one daughter cell; it may be generated after division, via information acquired from the cells' environment; or the distinct daughter cell fates may be achieved by a combination of these factors. A variety of cell divisions have been described in which asymmetry appears to be intrinsically determined (Horvitz and Herskowitz, 1992; Losick and Stragier, 1992; Poindexter, 1964; Strathern and Herskowitz, 1979). However, no factor has yet been shown to be both asymmetrically segregated to daughters and responsible for the eventual differences in their cell fates.

The differential segregation of gene products to daughters was first observed in the asymmetric divisions of the sea urchin embryo [Davidson, 1986 for review]. Similarly, in the first cell division of a *C.elegans* embryo, P granules segregate exclusively to the posterior daughter (Strome and Wood, 1983). In Caulobacter development, specific proteins and RNA localize to the poles of a predivisional cell and encode functions used by the daughter swarmer or stalk cell (Gober and Shapiro, 1991). Despite the identification of multiple gene products that are asymmetrically segregated, these genes have not been proven to determine the distinct fates of the daughter cells.

Conversely, multiple genes in *C.elegans* have been shown genetically to be required for the asymmetry of specific divisions (Bowerman et al., 1992; Ferguson et al., 1985; Sternberg and Horvitz, 1988). One of these gene products, skn-1, is expressed preferentially in one daughter of the asymmetric

division (Bowerman et al., 1993). However, neither skn-1 nor any other protein required for asymmetry of a division has been shown to segregate differentially upon division.

The *Drosophila* PNS is comprised of sensory structures that relay sensory information to the Central Nervous System [CNS] via peripheral neurons (Hartenstein, 1988; McIver, 1985; Zacharuk, 1985). One type of sense organ, the external sensory [es] organ, minimally consists of four cells: the neuron, the thecogen (sheath) cell which forms the innermost sheath around the dendrite of the neuron, and the trichogen (hair) and tormogen (socket) cells which are termed the "outer" support cells (Figure 1.1A). All four cells are descendants of a Sensory Organ Precursor [SOP] cell (Bate, 1978; Bodmer et al., 1989). The SOP, which is originally epidermal, delaminates and divides to produce two secondary precursor cells, labeled IIa and IIb in Figure 1.1B. The IIa cell then divides to produce the outer support cells, while the IIb cell divides shortly after its sister and forms the neuron and sheath cells (Figure 1.1B). All of the divisions described are asymmetric, in that each gives rise to two cells that have different fates.

Embryos mutant for *numb* contain es organs in which the neuron and sheath cells are replaced by two additional outer support cells (Uemura et al., 1989) (Figure 1.1C). Uemura et al. proposed that this phenotype may be the result of the disruption of asymmetry in the first division, yielding two equal secondary precursor cells that both give rise to outer support cells. We now demonstrate that the numb protein is asymmetrically localized within the SOP cell before division and is distributed primarily to one daughter cell upon division. In complementary experiments, we show that misexpression of the numb protein in both daughters of the SOP cell also disrupts the asymmetry of the SOP division, resulting in two equivalent cells that both give rise to neuron

MATERIALS AND METHODS

Drosophila Stocks

Flies were raised on standard cornmeal-yeast agar medium at 25°C. The following stocks were used in this study: *yw; Oregon-R* , *numb* ^{1} *pr cn Bc/Cyo*, *numb* ^{3} *pr cn Bc/Cyo*, (Uemura et al., 1989) *yw Flp; P[FRT]40A*, derived from the *yw Flp* and *P[FRT]40A* stocks (Xu and Rubin, 1993). The neurogenic alleles used are as follows: *Notch* $^{Df(N8)}$, *Delta* ^{9P39}, *mastermind*^{N2G}, *neuralized*^{Kx9}, *bigbrain*^{Fx1}, *Enhancer of split*^{8D06} . All alleles are described in Lindsley and Zimm, 1992. The *twist*^{IIH}*snail*^{IIG} *cn bw sp* stock, balanced over the *SM6B eve lac Z* chromosome, was given to us by S. Panzer. The *y*$^{+}$ *numb*^{3} *ck P[FRT]40A/CyO* chromosome used for mosaic analysis was generated by recombination between *numb* ^{3} *pr cn Bc* and *y*$^{+}$ *bib*^{Fx1} *ck P[FRT]40A*, which was originally constructed by D. Doherty.

Immunohistochemistry

Polyclonal antibodies against numb were raised in rabbits using the peptide: CKQLSPDLPIPSTARSPLARHSTNPFISPPK. This represents amino acids 517-546 in the sequence of the product of the zygotic numb transcript plus an added N-terminal cysteine. Antiserum was preadsorbed twice against 4-6 hour wild-type embryos at a 1:20 dilution in PBT (10mM Phosphate buffer, pH 7.0, .15M NaCl, .5% Triton X-100) +2%BSA+2%NGS. This preadsorbed serum was diluted further for immunohistochemistry to a final dilution of 1:1000 of the original serum. Preadsorbed guinea pig anti-asense antibody was obtained from A. Jarman and used at a dilution of 1:2000 of original serum. All other antibodies employed in this study have been described previously (Blochlinger et al., 1990a; Fujita et al., 1982; Vaessin et al., 1991).

Embryos were rinsed, dechorionated, fixed and devitellinized as described by Bodmer and Jan (1987). Following rehydration into PBT, embryos were blocked with PBT+2%NGS+2%BSA for one hour at room temperature. Primary antibody was added in the same blocking buffer and incubated overnight at 4°C. Embryos were washed one hour with four changes of PBT and incubated with secondary goat anti rabbit-HRP or goat anti-mouse-HRP for one hour. Avidin-Biotin amplification was used for rabbit anti-b-gal (Elite ABC kit, Vectalabs). Three rinses in PBT were followed by three rinses in 120mM Tris pH 7.6, at which time diaminobenzidine was added to 1mg/ml. Hydrogen peroxide was added at .0015% and the reaction was visualized under a dissecting microscope. Reactions were halted with rinses in ethanol. Embryos were cleared in toluene and mounted in permount.

Pupal nota were treated in essentially the same manner, except the fixation was carried out before dissection, by bathing the pupae in 4% formaldehyde in PBS for thirty minutes. Nota were mounted onto slides in Hoyer's solution (Ashburner, 1989).

Immunofluorescence and Confocal Microscopy

Embryo preparation and primary antibody incubation was the same as described for DAB reaction. Asense primary antibody that was generated in guinea pig was used for double labeling experiments. 2% Normal Donkey Serum was added to the fourth PBT wash. As secondary antibodies, fluorescein conjugated Donkey anti-Rabbit and rhodamine conjugated Donkey anti-guinea pig were added for a one hour incubation. In some cases, the asense signal was amplified, using biotinylated goat anti-guinea pig as a secondary antibody, followed by a one hour incubation with avidin conjugated to rhodamine. Embryos were washed with PBT three times and PBS once

before mounting in 90% glycerol with n-propyl-gallate to inhibit photobleaching. Confocal images were viewed and analyzed using a Zeiss microscope equipped with a BioRad Krypton/Argon laser (Bio-Rad).

Heat Shock experiments

The zygotic *numb* cDNA was cloned into the KpnI site of the pWHI vector plasmid (Blochlinger et al., 1991), which contains the hsp70 promoter. This hs-*numb* construct was introduced into *yw*, Oregon R embryos by P-element transformation (Spradling, 1986) with the helper plasmid pD2-3 (Laski et al., 1986). Thirty-two independent lines were established. For heat shocks, embryos were collected on grape agar plates that were placed in a 25°C moist chamber until embryos reached four to seven hours of age. The entire chamber was then submerged in a 39°C water bath for thirty minutes. Embryos were aged to stage 15-16 and fixed, or fixed within 1-2 hours. For pupal heat shocks, white prepupae were collected in vials and aged at 25°C. At the appropriate times, vials were placed in a 39°C water bath for forty five minutes. All data shown is with hs *numb* line #2.4C. However, effects of ectopic numb expression during pupal stages were detected in six of seven lines tested.

Western Analysis

Embryos were collected, aged and heat-shocked as described above. Following a two hour recovery period at room temperature, embryos were dechorionated with 50% bleach and rinsed several times with .7% NaCl .03%TritonX-100. Embryos were transferred to 1.5 ml eppendorf tubes, rinsed with phosphate buffer, pH 7.2, and homogenized in 100µl of 1x Laemmli loading buffer per 10µl of embryos. Non-heat-shocked control embryos were

processed in parallel. Embryo extracts were boiled for 10 minutes, centrifuged 5 minutes, and applied to an SDS-polyacrylamide gel at 5µl per lane.

After SDS-PAGE, proteins were transferred to nitrocellulose as described by Uemura et al. (1989). Immunoblotting was performed using numb antiserum at 1:1000 dilution and an HRP conjugated goat anti-rabbit secondary at 1:5000. Filter-bound antigen was detected using enhanced chemiluminescence ("ECL": Amersham).

Mosaic Analysis

numb mutant patches were obtained using the Flp FRT recombination system. These crosses were maintained at room temperature. Mosaics were generated in progeny of the following cross: *yw; y+ numb^3 ck P[FRT]40A/CyO* female X *yw hsFlp; P[FRT]40A/CyO* male. The Flp recombinase was induced in the progeny by administering two forty-five minute heat pulses at 39°C with 45 minutes intervening rest. Heat pulses were given at first, second, or third instar larval stages. Flies of the following genotype were examined for mutant patches: *yw hsFlp; y+ numb^3 ck P[FRT]40A/P[FRT]40A*. Male progeny, which contain no *hs Flp*, were analyzed as a control.

RESULTS

NUMB EXPRESSION PATTERN

Numb is localized in the Sensory Organ Precursor and is asymmetrically distributed to daughter cells

The pattern of numb protein expression was examined using a polyclonal rabbit antibody raised against the last 30 residues of the numb protein sequence (Uemura et al., 1989). In order to determine if the SOP cell expresses numb, these embryos were also labeled with antibody against the asense protein, which appears in the SOP after it becomes specified (Brand et al., 1993a; Campuzano and Modolell, 1992).

For simplicity, fully germ band extended embryos (stage 11, (Campos-Ortega and Hartenstein, 1985) were analyzed. At this stage there are only two asense expressing PNS cells per abdominal hemisegment, the anterior [A] and posterior [P] cells (Brand et al., 1993a). The anterior cell gives rise to the first external sense organ of the hemisegment, the doubly innervated trichoid sensillum. The posterior cell is a precursor for the lateral chordotonal organs. At this stage, numb expression during the division of the "A" cell can be monitored without being obscured by the later preponderance of asense staining PNS cells.

In the "A" cell, we found anti-numb immunoreactivity in a ring along or closely associated with the plasma membrane, with enrichment in a portion of the ring, generating a crescent pattern (Figure 1.2A). These results suggest that numb protein is associated with the plasma membrane and is asymmetrically localized. Because asense is a DNA binding nuclear protein (Brand et al., 1993a; Jarman et al., 1993b), its cytoplasmic localization within a cell is indicative of the stage of the cell cycle. For example, in the "A" cell shown in Figure 1.2B, asense is cytoplasmic, suggesting that this cell is in

mitosis, and breakdown of the nuclear membrane has occurred. Figure 1.2C merges the images in 2.2A and 2.2B. In the majority of cases, we detect crescents associated with cells that are in mitosis.

We find, however, that a low proportion of mitotic "A" cells express numb. We believe that this reflects the close timing of numb localization and the completion of cell division.

To facilitate the visualization of SOP cells expressing numb, we examined protein distribution in SOP cells from the adult PNS. During development of the notum microchaetae in the adult, numerous es organ precursors of approximately the same developmental stage can be analyzed (Hartenstein and Posakony, 1989). In this case, we can detect many predivisional SOP cells that express the numb protein asymmetrically. When examining a field of SOP cells, however, we also detect cells that are in the process of cytokinesis. Figure 1.2(D-F) shows one such SOP cell. Note that numb staining is predominantly in the membrane surrounding one of the daughter cells. Although we cannot identify which daughter receives numb, it is clear that the protein is differentially distributed to one daughter cell. The importance of numb in adult es organ formation will be discussed later.

Numb is segregated to the Ganglion Mother Cell during the division of neuroblasts

The assertion that numb protein is differentially distributed to the daughter cells of an asymmetric division is further substantiated by the expression pattern observed for the CNS. The division of a neuroblast, like that of the SOP cell, is asymmetric, forming a small ganglion mother cell [GMC] and another large neuroblast (Figure 1.3C) (Bauer, 1904; Doe, 1992). Numb protein appears in the portion of the neuroblast membrane that faces

the interior of the embryo (Figure 1.3A, arrow). The image in 3B captures a neuroblast in the midst of division, demonstrating differential segregation of the protein to the GMC. Numb protein persists in the GMC until shortly after division (Figure 1.3A and B, open arrows).

In addition to expression in the nervous system, *numb* is detected in the membrane of epidermal cells, where it is not localized asymmetrically (data not shown). Epidermal expression peaks during gastrulation (stage 6-7), subsides during neurogenesis, and resumes as PNS cells proliferate and differentiate, at about stage 13. We have not found epidermal defects in *numb* mutant embryos. Although *numb* mutant embryos display patterning defects in a small subset of muscles (Uemura et al., 1989), we do not observe expression in the mesoderm.

Numb localization is independent of signals from mesoderm and ectodermal cells

Because the numb protein localizes to the side of the cell adjacent to the mesoderm, we asked whether, in the absence of mesoderm, numb can still localize within the neuroblast. We examined numb expression in embryos double mutant for *twist* and *snail*, which direct mesoderm formation (Anderson, 1987; Leptin and Grunewald, 1990). Despite the lack of mesodermal tissue in these embryos, the neuroblasts display the crescent pattern of numb localization that, in wild-type, prefigures asymmetric distribution of numb to the GMC. (Figure 1.3D).

In order to determine if proper numb localization relies on signaling from neighboring ectodermal cells via the neurogenic genes, we labeled embryos mutant for *Notch, Delta, neuralized, mastermind, bigbrain* or *Enhancer of split* (for reviews of neurogenics, see (Artavanis-Tsakonas, 1988; Artavanis-

Tsakonas and Simpson, 1991; Campos-Ortega and Jan, 1991; de la Concha et al., 1988) with anti-numb antibody. Figure 2E shows a *Notch* embryo, in which the numb staining is representative of the pattern observed in the other neurogenic mutant embryos: The asymmetric distribution of numb to daughters, identified by the crescent staining pattern, is preserved during neuroblast division.

ABSENCE OF NUMB AND MISEXPRESSION OF NUMB GIVE RECIPROCAL PHENOTYPES

The observation that numb protein segregates differentially to daughter cells suggests that *numb* may be necessary to give IIa and IIb distinct fates, that is, to generate asymmetry in this division. The *numb* mutant phenotype has been interpreted as a transformation of the IIb cell to another IIa cell (Uemura et al., 1989). We wondered whether the converse was true, that is, whether misexpression of numb in all cells causes the reverse transformation, forming two IIb cells.

To address this question, we constructed a P-element vector that expresses the *numb* zygotic cDNA under the control of the heat shock inducible hsp 70 promoter (Figure 1.4A). The hs-*numb* construct was injected into embryos and independent transformant lines were obtained. We tested embryos from these lines for numb protein induction following a brief heat shock at 39°C. In whole mount preparations of the heat-shocked embryos, we detected increased amounts of numb protein in the epithelial and neuronal cell membranes (Figure 1.4D). Curiously, the crescent pattern is sometimes still detectable in neuroblasts (see arrow) indicating that the machinery for localization is functioning. However, the elevated level of general numb expression throughout the cell membrane suggests that the machinery of

localization can be saturated. Western analysis of embryonic extracts made from these hs-*numb* lines shows a several fold induction of the 61 kD numb protein over wild-type levels (Figure 1.4B). The ability of our antibody to recognize the product of numb cDNA on a western blot and the lack of neuroblast staining in *numb* homozygous mutant embryos (Figure 1.4C) confirm that the antibody is specific to the numb antigen.

Misexpression in the embryo transforms outer support cells to neuron and sheath cells

In order to test the prediction that expression of numb in both daughters of the SOP cell would result in two IIb cells, leading to duplication of neurons and sheath cells, we induced numb in hs-*numb* embryos at a time when the SOPs divide, 5-9 h of development at 25°C (Bodmer et al., 1989; Ghysen and O'Kane, 1989). After heat shock, the embryos were returned to 25°C until the completion of PNS development. We then fixed and stained the embryos with a variety of antibodies that recognize specific cells in the PNS.

We limited our analysis to the dorsal-most abdominal es organs, which are adequately separated from the rest of the dorsal es cluster, facilitating the identification of their constituent cells. Figure 1.5A shows two hemisegments from a wild-type embryo that has been subjected to heat shock, aged to stage 16, and stained with both MAb22C10 and antibody against prospero. MAb22C10 recognizes neuronal cell bodies, dendrites and axons (Canal and Ferrus, 1986; Hartenstein, 1988; Zipursky et al., 1984), while prospero antibody identifies nuclei of sheath cells [sh] (Figure 1.5A, open arrow)(Vaessin et al., 1991). The dendrite [d] of the dorsal-most es organ in the hemisegment on the left is indicated by an arrow.

Figure 1.5B shows two hemisegments from a hs-*numb* embryo in which the left hemisegment has a transformed es organ. As predicted from the model, this transformed es organ has a duplicated neuron that projects a dendrite parallel to the original dendrite (arrows). The double staining with antibody against prospero demonstrates that a sheath cell associates with each neuron (open arrows), indicating that both the neuron and sheath cells are duplicated. In this particular hemisegment, the es organ just ventral to the dorsal-most organ is also transformed. Our heat shock induction conditions yield dorsal es transformations in one to three hemisegments per embryo.

The extra neuron and sheath cells could arise from additionally formed cells, or they could result from the transformation of existing cells from their normal fates to that of neuron and sheath. If this phenotype is indeed the result of a cell fate transformation, we predicted that the extra neuron and sheath cell would have arisen at the expense of the outer support cells. To test this possibility, we stained hs-*numb* embryos with antibody against the Cut protein. This antigen is nuclear and is present at higher levels in the outer cells of the es organ (the socket and hair cells) than the inner cells (the neuron and sheath)(Blochlinger et al., 1990a).

In hs-*numb* embryos, we observe es organs with two neurons, but no strongly staining outer cells. For example, Figure 1.5C displays two abdominal hemisegments in a hs-*numb* embryo that has been double stained with MAb22C10 and anti-Cut. In the segment on the right, a single dendrite projects to meet the two outer support cells [osc] that are strongly Cut positive. This untransformed segment assures us that Cut expressing outer cells are clearly visible at this stage. The segment to the left shows an es organ with an extra neuron (arrows point to parallel dendrites), and no Cut expressing outer cells can be seen (dotted rectangle). The absence of outer cells in these

organs which have duplicated neuron and sheath cells suggests that the outer cells have been transformed to inner cells. This transformation, caused by misexpression of *numb*, is opposite to the transformation observed for es organ cells in the *numb* loss of function mutant.

Extra neurons were also observed in the chordotonal [ch] organs, the cells of which are also derived from a common precursor cell (Bodmer et al., 1989). The proposed lineage for ch organ cells is diagrammed in Figure 1.6A. Figure 1.6B shows two hemisegments in a lateral view of a heat-shocked wild-type embryo. The v' ch neuron [n] is in focus, and the open arrow identifies the associated sheath cell [sh]. Induction of ectopic numb expression causes two detectable phenotypes in the v' ch organs: In the first case (Figure 1.6C), both the neuron (arrows) and the sheath cell (open arrows) are duplicated, as revealed by MAb22C10 and prospero antibody. *numb* loss of function mutant embryos display a transformation of neuron and sheath cells to cap cells, suggesting that numb may act in the second division of the ch lineage (Uemura et al., 1989). Although we have not confirmed a loss of cap cells concomitant with the emergence of the extra neuron and sheath, the heat-shock induced phenotype could be opposite to the transformation observed for *numb* loss-of-function. The second observed transformation is illustrated in the two hemisegments in Figure 1.6D. Two v' neurons are detected which have no associated sheath cells (arrowheads). Without their sheath cells, these neurons fail to extend dendrites. This phenotype can be interpreted as a transformation from sheath cell to neuron and indicates a defect in the final division. Both of these fate transformations are reminiscent of the es organ cell transformation in that they affect asymmetric divisions.

In contrast to the lower frequency detected for es organ transformations, cells of the v' ch organ are transformed in 50-90% of hemisegments.

FUNCTION OF NUMB IN ADULT ES ORGANS

We have analyzed the role of *numb* in formation of adult es organs, which undergo the same division pattern as their analogs in the embryo (Hartenstein and Posakony, 1989). The adult provides two advantages over the embryo: First, the spatial arrangement of the organs allows unequivocal identification of the cells of a single organ, and second, the cell divisions occur over the span of several hours, enabling experimental separation of the first and second divisions (see diagram in Table 1.1). Precursors of the microchaete bristles appear at about 14 hours After Puparium Formation [APF]. The precursors divide to form the IIa and IIb cells at about 15-16 hours APF (Hartenstein and Posakony, 1989). The outer support cells derive from the division of the IIa cell, which divides at about 17 hours APF. The IIb cell divides about an hour later, giving rise to the neuron and sheath cells. As described above, numb protein is asymmetrically distributed in the SOP cells that give rise to the microchaetae, providing further evidence that adult es organs develop in a manner analogous to the embryonic sense organs.

Ectopic expression of numb in adults yields two types of transformations

In order to look for effects of hs-numb at the various divisions in microchaete formation, numb was induced at different times within the range of 12-24 hours APF. The heat-shocked pupae were allowed to develop until adulthood and analyzed. Numb induction promotes two distinct phenotypes: Balding and twinning of bristles (Figure 1.7B,C). The phenotype induced in a

given fly depends upon the time of heat shock application. We examined flies that were heat-shocked within a two hour interval and determined the percentage of flies showing the phenotype within each two hour interval. Table 1.1 displays the results from one representative experiment. The peak interval of sensitivity to balding effects is 16-18 hours APF. This corresponds to the two or early three cell stage of microchaete development. The twinning effects appear to peak when heat shock is administered 18-22 hours APF. This period corresponds to the three or four cell stage.

We have observed the twin and bald phenotypes in various other bristles on the adult fly, including macrochaetae, sternital and tergital abdominal bristles, leg bristles, wing margin bristles and bristles within the eye. Whether the twinning and balding is associated with any cell fate alterations of the neuron and sheath cells is examined below (mainly for the microchaetae). In addition to these two predominant transformations, we also detect twin hairs with a socket and multiple small hairs emanating from one socket at a very low frequency.

Early numb induction causes transformation of outer cells to inner cells

The "balding" phenotype could reflect the absence of the entire bristle or the absence of the outer support cells that form the external sensory structure. To distinguish between these possibilities, we looked for the presence of neurons under the bald patches of 43h APF nota using MAb22C10. We found that two neurons per organ underlie the "bald" area, analogous to the transformation described for the embryonic organs after heat shock (Figure 1.7E). We have attempted to identify sheath cells in hs-*numb* pupal nota, but we find that the prospero antigen is absent in wild-type adult es organs. In the absence of sheath cell markers, we can only extrapolate from

the embryo and postulate that duplicated sheath cells associate with the duplicated neurons. In addition, we observe cells in some of these bald nota that appear to be the result of partial transformation of outer cells to inner cells. Although these cells reside subcutaneously, they seem to produce cuticle that has the morphological appearance of hair and socket like cells (Figure 1.7G,H).

Late induction causes transformation of socket cell to hair cell

The twinning phenotype appears to reflect the formation of an extra or "twin" hair cell at the expense of the socket cell. This transformation occurs less frequently than the transformation that yields the bald phenotype (at most, about 20% of microchaetae per notum are transformed). It is therefore necessary to dissect and stain for neurons after the four cells differentiate completely (so that twin hairs can be recognized). Since the balding and twinning phenotypes are also observed for macrochaetae, and in our hands, staining of the macrochaetae at this stage is more reliable than microchaetae staining, we labeled twinned macrochaetae with MAb22C10 (Figure 1.7F). We observe a single neuron innervating this structure. Thus, in this case, the IIa cell lineage is affected by the induction of numb, whereas the IIb cell lineage appears normal . Conversely, we also observe bristles that have normal outer cells and two neurons, implying the transformation of sheath cell to neuron in the IIb lineage may also occur (data not shown). Since the IIa cell divides before the IIb cell, it is not surprising that the two divisions can be altered independently.

numb mutant patches in mosaics contain abnormal bristles

The three existing alleles of *numb* are embryonic lethal; therefore, no adult role for *numb* has yet been described. In order to uncover a requirement for *numb* in later stages of development, we generated mosaic animals that contain patches of homozygous *numb* mutant tissue in a heterozygous *numb* background. Mosaics were induced using the Flp/FRT method (Golic and Lindquist, 1989; Xu and Rubin, 1993) in flies of the following genotype: *yw*Flp; y^{+}*nb ck P[FRT]40A/P[FRT]40A*. Homozygous *numb* patches were thus marked with the cell autonomous mutant, *crinkled* [*ck*], which causes epidermal hairs to appear smaller and multiplied. We found that bristles that are within a ck^{-} patch develop abnormally. Figures 1.8A-C depict the range of phenotypes observed for $numb^{-}$ macrochaetae. In all cases, the hair cell fails to form and at least three, though generally four sockets are clearly discernible. To determine if neurons are associated with these sockets, we dissected and stained mosaic nota with MAb22C10. In 60 of 61 mutant macrochaetae examined, we could not detect a sensory neuron associated with the abnormal sockets (Figure 1.8D, asterisk).

In the case of microchaete bristles, loss of *numb* function yields a broader spectrum of transformations. Although the four socket phenotype is detected in approximately 10% of mutant microchaetae (Figure 1.8E, stout arrow), most microchaetae show milder defects. Some of these bristles form a remnant of a hair cell associated with multiple socket-like cells (open arrow). Most mutant microchaetae form hair cells that have the coarse *crinkled* phenotype, although cuticular abnormalities at the base of the hair cell indicate that the loss of *numb* has some effect on these bristles (curved arrow). The ck^{-} microchaetae are found both on borders and within large mutant patches, suggesting that the mild phenotype is not the result of rescue by neighboring $numb^{+}$ cells.

The embryonic mutant phenotype predicts that adult *numb*$^-$ bristles should have four outer cells and no neuron or sheath cells. Since Cut antibody was used to identify extra outer cells in embryonic analysis (Uemura et al., 1989), no distinction was made between the hair and socket cells. Although our initial expectation was to see two sets of hair and socket cells (see Figure 1.1C), the twin phenotype resulting from misexpression suggests that *numb* may also be involved in the fate decision between hair and socket cells. Incorporating this information, we propose that the four sockets are the result of loss of *numb* function at two steps in the development of the es organ. The first step is at the secondary precursor level, such that loss of *numb* yields two IIa cells. The second requirement for *numb* is in the differentiation between hair and socket cells. Mosaic patches, in which both activities of *numb* are eliminated, would be predicted to and do yield four socket cells (Figure 1.9C).

DISCUSSION

asymmetric distribution of numb protein at division specifies the asymmetric fates of SOP daughters

We have described the role of the gene, *numb*, in generating two distinct daughter cells upon division of the SOP cell. *numb* has been shown to be essential for proper fate specification of the neuron and sheath cells, which are descendants of the IIb secondary precursor cell (Uemura et al., 1989). In *numb* mutant embryos, these cells differentiate inappropriately, forming a supernumerary pair of outer support cells, normally descendants of the IIa cell. By immunocytochemistry, we demonstrate that numb is asymmetrically localized in the SOP cell before division, then is predominantly partitioned to one daughter cell. We show that ectopic expression of numb from a heat shock inducible promoter causes a change of fate from outer cells into inner cells. We therefore propose that numb acts to generate distinct secondary precursor cells: we interpret the null mutant phenotype as a transformation of IIb to IIa, forming two IIa cells; conversely, we propose that the embryonic es phenotype and the adult balding phenotype that result from the misexpression of *numb* reflect a transformation of IIa to IIb, yielding two IIb cells (Figure 1.9A). This is the first account to our knowledge of an asymmetrically segregated gene product that determines the asymmetric fates of the daughter cells. These results further demonstrate that fate determination via the asymmetric distribution of proteins is not limited to early embryonic divisions. Finally, our results show that lineage, or the information inherited by a cell, has a role in the specification of es organ cell fates.

numb acts in multiple asymmetric cell divisions

We have found that *numb* acts at multiple divisions in the sensory organ lineage. Induction of hs-*numb* after the second round of division results in a duplication, or twinning, of hair cells, usually at the expense of the socket cell (Figure 1.9B). Conversely, *numb* mutant patches in an otherwise wild-type fly display a loss of hair cell and abnormal or multiple sockets. As in the SOP case, the loss of function and misexpression phenotypes represent the two different fates that the daughter cells normally acquire. Thus, the asymmetric division of the IIa cell in the adult es organ lineage also appears to rely on *numb* function. We believe that *numb* may act in the IIb cell lineage as well (M.S.R., unpublished results). This implies that numb is newly expressed in both IIa and IIb before division and distributed asymmetrically to their daughter cells.

The second division in the chordotonal organ lineage was previously shown to be affected in *numb* null mutants such that extra cap cells form at the expense of the neuron and sheath (Uemura et al., 1989). This phenotype could be interpreted as a transformation of the precursor cell that normally forms a neuron and sheath cell into its sister, the cap cell. In this report, we show that the misexpression of numb from a heat shock promoter leads to the production of additional neuron and sheath cells. This data is consistent with the model that misexpression of numb yields a transformation that is opposite to the loss of function transformation. We cannot identify the precursor [labeled chII in Figure 1.6A] to the neuron, sheath and cap cells, but we hypothesize that numb may be asymmetrically localized in precursor chII and differentially distributed to its daughters, conferring distinct fates upon them. We have also described how the misexpression of numb transforms the sheath cell to neuron in the chordotonal organ. Because loss of function mutants apparently lack the precursor for the neuron and sheath cell, it is not

possible to determine whether numb plays a role in this division without embryonic mosaic analysis. It is useful, however, to consider that this may be another division in which the ultimate asymmetry results from the asymmetric distribution of numb protein.

Although the differential segregation of the numb product to one daughter perhaps can best be seen in dividing CNS neuroblasts, we have not characterized whether all or merely a subset of neuroblasts express numb asymmetrically. We do not see a gross defect in the CNS corresponding to the loss of *numb* function. However, Higashijima has observed alteration of Bar H1/BarH2 expression in the CNS of *numb* mutant embryos (personal communication). We note that any role for *numb* in the CNS may be hidden by the potential presence of residual maternal product in the embryos tested.

The variety of asymmetric divisions that require numb activity suggests that *numb* does not direct es neuron fate per se. Rather, *numb* appears to be involved in a general mechanism that is used in cases where distinct daughter cell fates are required.

numb and other genes involved in es organ cell fate

The analysis of hs-*numb* effects in the adult enabled us to dissect with more precision when *numb* acts. These experiments address the important question of whether *numb* is involved in the establishment or propagation of asymmetry. The timing of the heat shocks that cause transformations indicates that the action of *numb* is probably around the cell division, possibly in the daughter cell. The observation that misexpression at the secondary precursor stage induces identical fates in these sister cells implies that the membrane associated protein, numb, is likely to modulate a signaling pathway in the cell(s) that acquire it. We do not yet know how numb transmits a signal;

the protein sequence of numb is not significantly homologous to any known protein. However, a few candidate genes that may function in the same pathway have been identified.

A mutation in the *twins* gene gives rise to chaetae that have two sets of hair and socket cells and no neuron or sheath cells (Shiomi et al., 1994). This phenotype suggests that *twins*, like *numb*, is also required for determining IIb cell fate. Twins has homology to a regulatory subunit of Protein Phosphatase 2A. Another gene that affects secondary precursor fates is *oversensitive* [*osn*], which may antagonize *numb* function. Loss of function mutants in *osn* appear to cause a transformation from outer cells to neuron and sheath. (M. Guo, submitted).

In addition to *numb*, two genes have been described that affect the fates of the IIa cell descendants. *Hairless* hypomorphic alleles exhibit varying degrees of transformation from hair to socket cells (Bang et al., 1991; Lees and Waddington, 1942). Loss of function mutations in the *Suppressor of Hairless* [*Su(H)*] gene act as dominant suppressors of this phenotype, while a gain of function mutation in *Su(H)* enhances the *Hairless* phenotype (Ashburner, 1982; Lindsley and Zimm, 1992; Nash, 1965; Nash, 1970). *Hairless* encodes a novel basic protein (Bang and Posakony, 1992; Maier et al., 1992), while *Su(H)* is homologous to the mouse gene encoding the Recombination signal Binding Protein [RBP] (Schweisguth and Posakony, 1992). Given that misexpression of *numb* late in adult es organ development causes a socket to hair transformation, both of these genes are good candidates for genes that act in a pathway downstream of *numb*.

We stress that the identification of *numb* as an inherited component in cell fate determination does not preclude the involvement of cell-cell interactions, either in the establishment of numb protein asymmetry, or in

subsequent signaling. In fact, analyses of temperature sensitive alleles of *Notch* (Hartenstein and Posakony, 1990) and *Delta* (Parks and Muskovitch, 1993) indicate that the mechanism of lateral inhibition may be utilized during specification of the component cells of the adult es organ. For example, *Notch-ts* flies that have been shifted to the non-permissive temperature after SOP division produce abnormal sense organs that contain more than one neuron, often at the expense of the accessory cells. Such inhibitory interactions could theoretically occur at the two, three, and/or four cell stages. Furthermore, the demonstration that *BarH1* and *BarH2* (Higashijima et al., 1992) are expressed in the neuron and sheath cells, but are required for the proper differentiation of the campaniform type of outer support cells, demonstrates that signaling does occur between the inner and outer cells. It will be interesting to determine how these mechanisms interface. For example, the initial difference in numb concentration between the IIa and IIb cells may trigger a pathway in one cell that activates a ligand for subsequent cell-cell communication. Alternatively, cell intrinsic and extrinsic information might contribute fate information in parallel pathways that ultimately join to activate specific fates. This model, which invokes some redundancy, is consistent with the observation that *numb* mutant microchaetae do not always form four sockets. Similarly, redundancy in determination of es versus ch organ fate has been reported for mosaic patches that are mutant for null alleles of *cut* (Bodmer and Jan, 1987a).

how might the asymmetric localization of numb be established?

The observation that numb is localized in neuroblasts but not in epithelial cells implies that numb distribution does not strictly respond to the apical/basal polarity of the epithelial cell. The neuroblast, then, must acquire

the capacity to localize numb. Although we have not determined which factors direct this localization, we have eliminated some obvious candidates. The characteristic crescent pattern of numb localization in CNS neuroblasts is still achieved in embryos that lack mesoderm. This result is consistent with a previous demonstration that the mesoderm does not induce neural development in *Drosophila* embryos (Rao et al., 1991). The normal segregation of numb to the GMC is also visible in embryos mutant for the neurogenic genes, implying that the establishment of numb pattern is independent of the signaling pathway that selects the neural precursor cell from a cluster of equipotent cells. Since neurogenic mutants have no ventral ectoderm and *twist snail*, double mutants have no mesoderm, the information that directs the asymmetric localization of numb is probably intrinsic to the neuroblast. Therefore, we expect that the specification of the neuroblast initiates a pathway, independent of the neurogenic signalling pathway, that leads to the localization of numb. We hypothesize that this pathway relies on residual directional information retained by the neuroblast, which was originally a polar epithelial cell.

Figure 1.1. Diagrams of es Organ and Cell Lineages

(A and B). See legend for Figure 1 (C) In the *numb* mutant, the neuron and sheath cells are transformed into cells that make the outer structures. Although the extra cells are labeled hair and socket in this diagram, it cannot be determined from the marker used which type of outer support cells are formed. The transformation is proposed to be at the level of the secondary precursors, where IIb mimics the fate of its sister, the IIa cell.

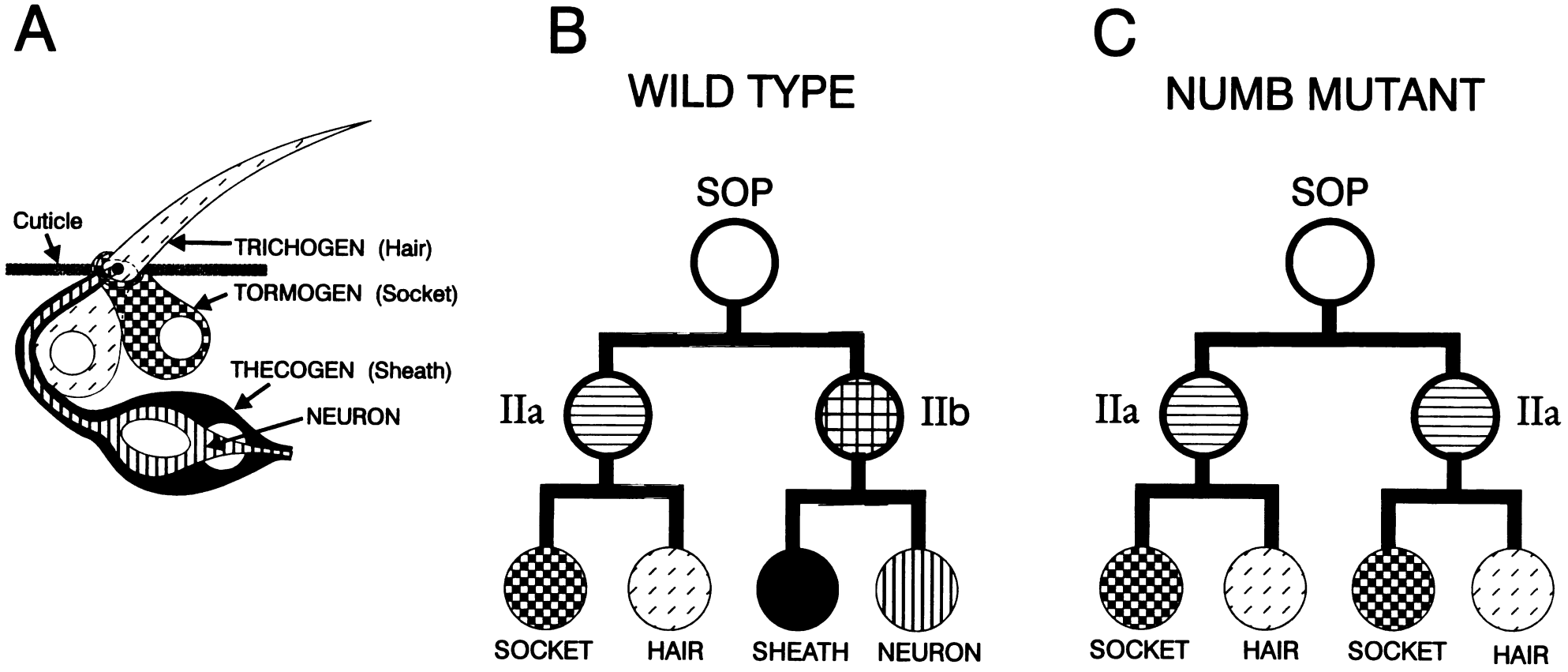

A
Cuticle
TRICHOGEN (Hair)
TORMOGEN (Socket)
THECOGEN (Sheath)
NEURON
B
WILD TYPE
SOP
IIa
IIb
SOCKET
HAIR
SHEATH
NEURON
C
NUMB MUTANT
SOP
IIa
IIa
SOCKET
HAIR
SOCKET
HAIR

Figure 1.2. Distribution of numb Protein in SOP cells

(A-C) Abdominal hemisegment from a 5 to 6 hour *yw* embryo. "A" and "P" designate the cells originally described by Ghysen and O'Kane (1989), that are also detected with asense antibody (A. Jarman, personal communication; Brand et al., in press). (D-F) A dividing SOP cell from a notum dissected at 14-16 h APF. This SOP gives rise to a microchaete bristle in the adult. (A and D) Views of the numb expression pattern. (B and E) Views of the asense expression pattern. (C) Superimposition of A and B to show that the crescent-shaped numb pattern corresponds to the membrane of the "A" cell. (F) Superimposition of D and E to show that numb expression is limited to one daughter in the dividing SOP cell (arrow).

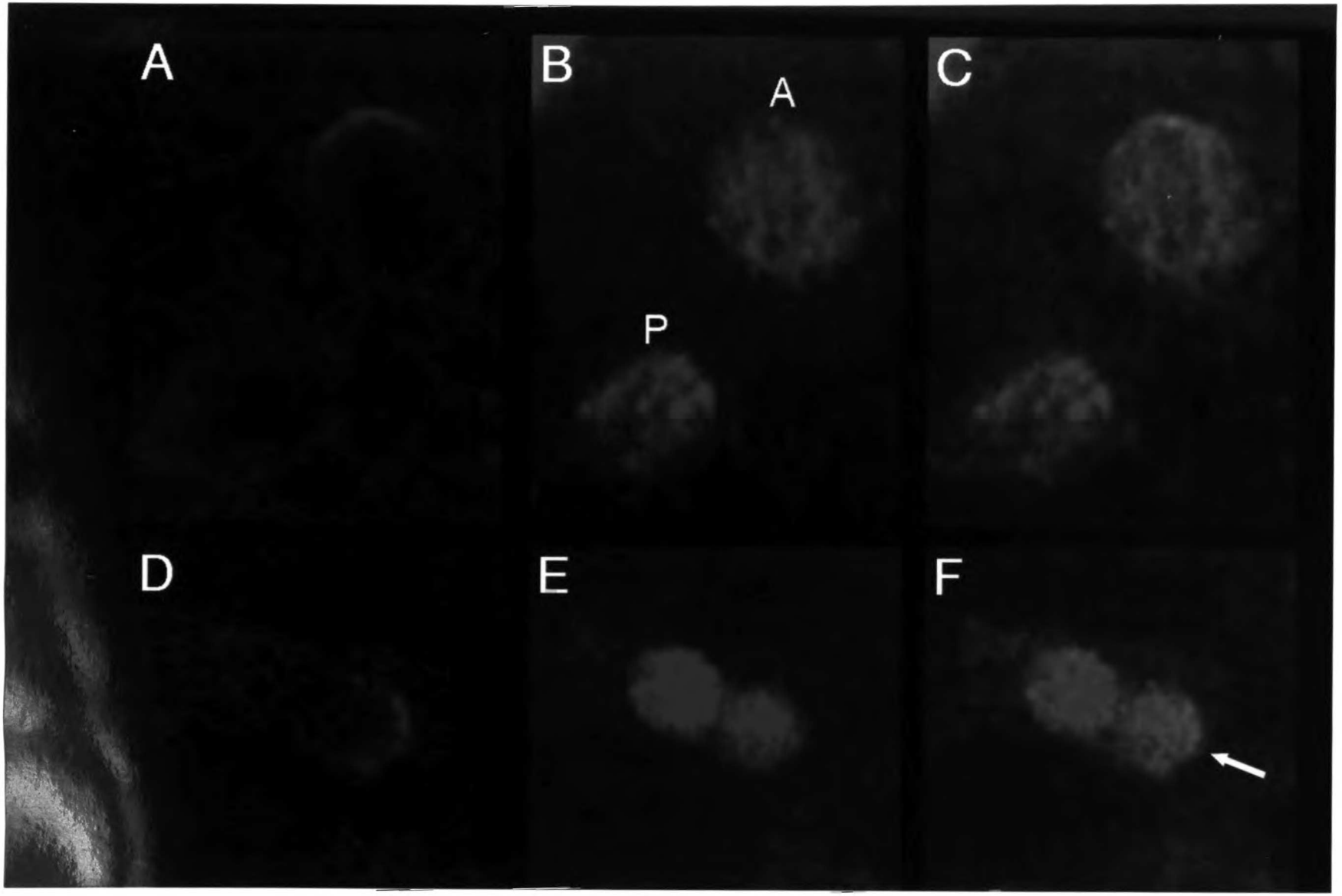

A
B
A
P
C
D
E
F

Figure 1.3. numb Protein Distribution in Neuroblasts from Wild Type, *twist snail* and *Notch*, Embryos.

(A) Numb antibody labeling of a wild-type, stage 10 embryo reveals that the protein is expressed in the neuroblasts (arrow) and Ganglion Mother Cells [GMC](open arrow). (B) Higher magnification of a numb-labeled wild-type embryo shows several neuroblasts at different stages of division. As the bud of a new cell forms, numb segregates predominantly to the bud (arrowhead). Just out of focus, a GMC that expresses numb can be seen. (open arrow). (C) A schematic diagram depicts the localization of numb during a division of a neuroblast [NB] cell, and its eventual distribution to the GMC. numb protein is represented by cross-hatched region. (D) In numb labeled (stage 10) *twi*$^{-}$*sna*$^{-}$ embryos (that lack mesoderm) crescent localization of numb in neuroblasts is still observed (arrow). (E) An example of a neuroblast in a *Notch*$^{-}$ embryo (stage 10) labeled with numb antibody indicates that, despite the distinctly epidermal position of the neuroblast, numb segregates properly to the GMC.

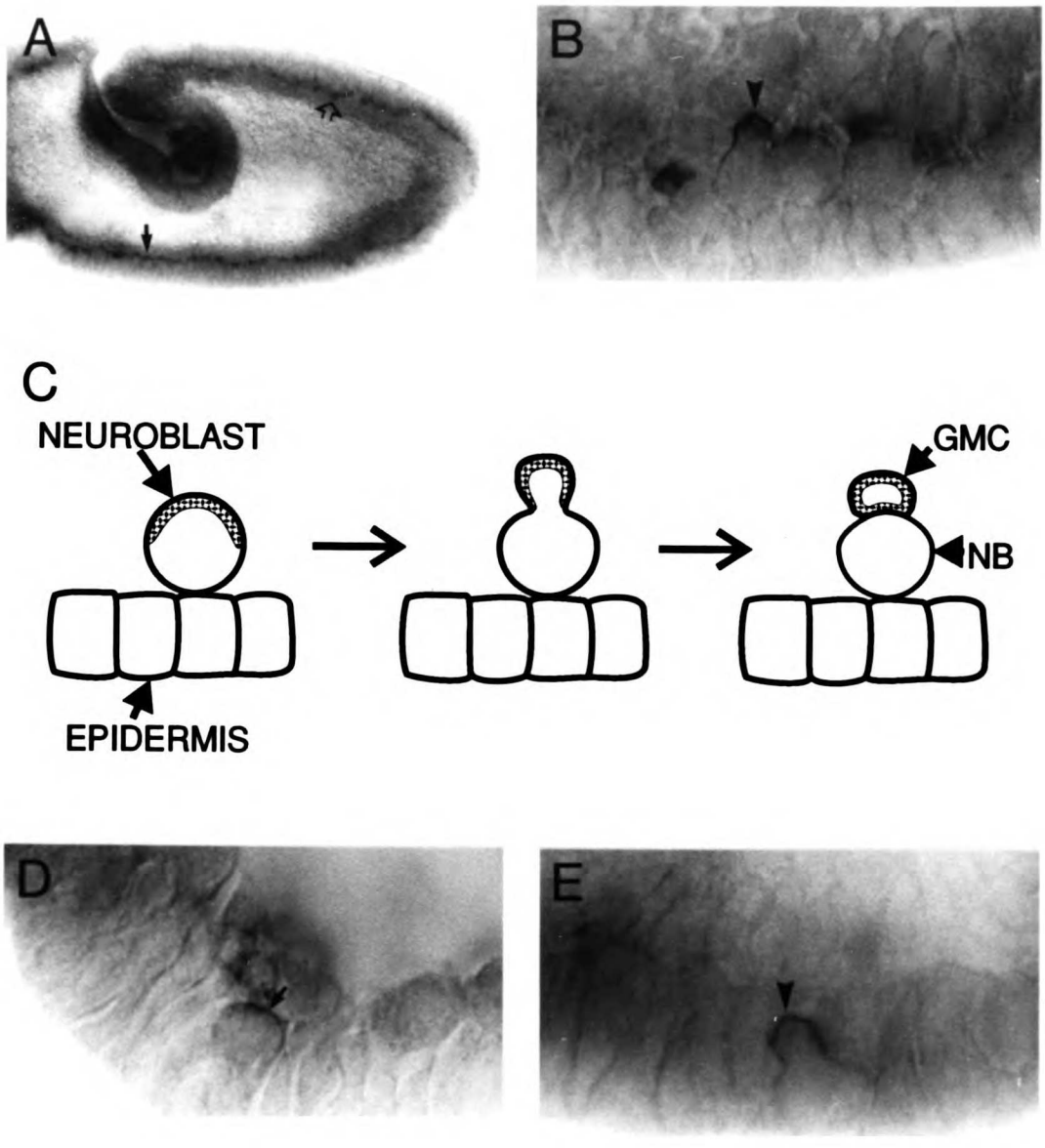

A
B
C
NEUROBLAST
GMC
NB
EPIDERMIS
D
E

Figure 1.4. The hs-*numb* Construct Causes Ectopic Expression of numb in Embryos

(A) Diagram of the hs-*numb* construct, containing *numb* zygotic cDNA (diagram is not to scale). (B) Western blot analysis with anti-numb antibodies of embryonic extracts from 6-9 hour wild-type (lane 1), wild-type heat-shocked (lane 2), uninduced hs-*numb* (lane 3) and induced hs-*numb* (lane 4) embryos. The amplification of two bands in the 60 kD range indicates that the extra band does not represent the maternal product as previously suggested (Uemura, 1989) but instead represents a modified form of the numb protein. (C) A *numb*^{1}*pr cn Bc* homozygous mutant embryo, labeled with numb antibody has no staining in epidermis or neuroblast, showing specificity of the antibody. (D) A view in the plane of the neuroblasts in a hs-*numb* embryo labeled with anti-numb antibodies 1 hour after heat shock treatment. Compare with wild-type expression in figure 3A: Staining of the wild-type and hs-*numb* embryos shown were done in parallel and developed for the identical length of time. Despite the induction of numb throughout the neuroblast and epidermal layers, some asymmetry can still be detected in the neuroblasts of hs-*numb* embryos (arrow).

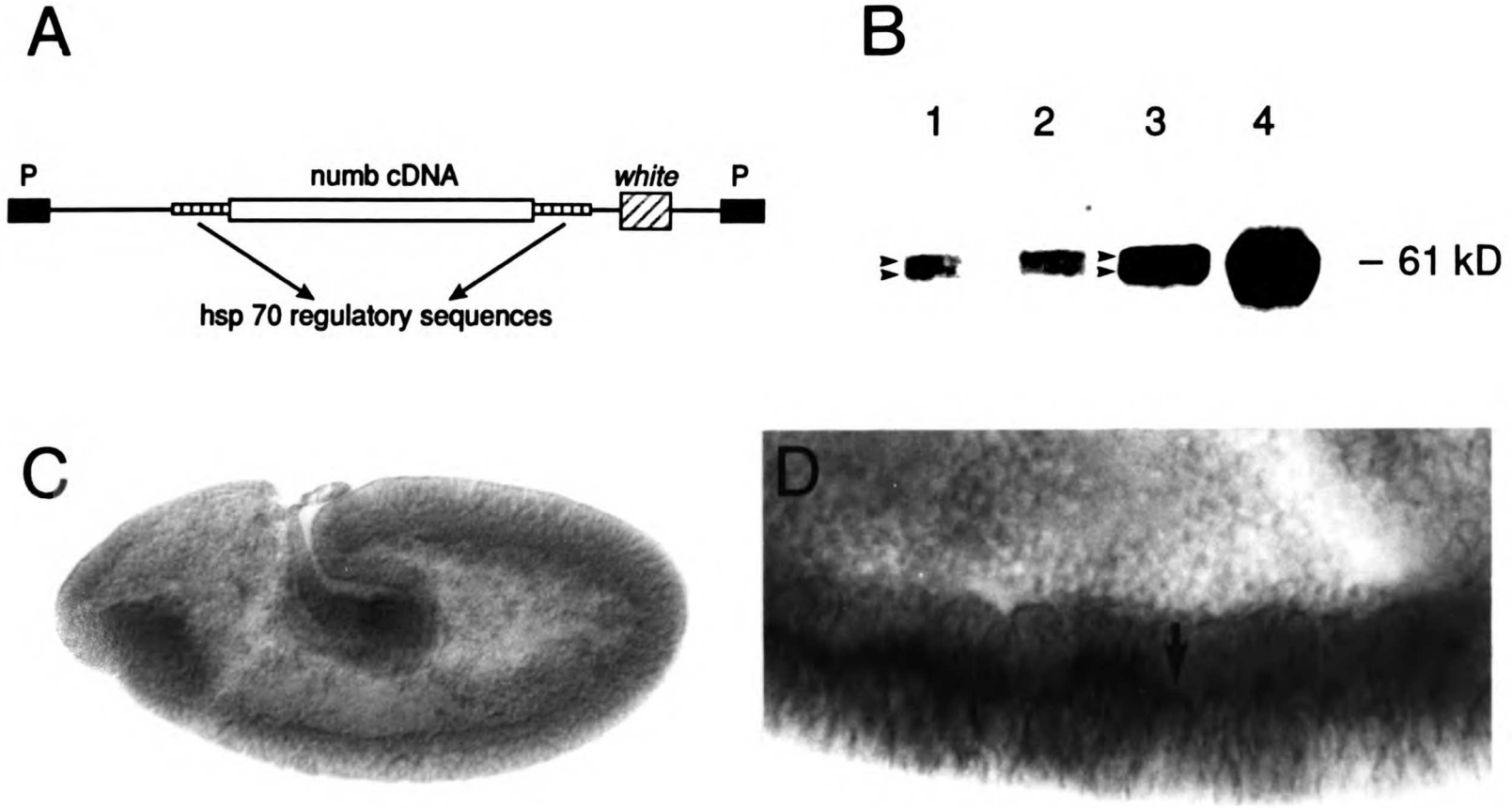

A
P
numb cDNA
white
P
hsp 70 regulatory sequences
B
1
2
3
4
– 61 kD
C
D

Figure 1.5. Transformation of Hair and Socket Cells into Neuron and Sheath Cells in hs-*numb* Embryos

Views of two dorsal-most es organs in two abdominal hemisegments of a wild-type (A), and hs-*numb* (B,C) embryo (stage 16) subjected to heat shock treatment (The dorsal-most es organ corresponds to desD in the nomenclature of Ghysen et al., 1986). Embryos in A and B are double labeled with anti pros, which identifies sheath cell nuclei [sh] and MAb22C10, which defines neuron and their dendrites [d]. The hemisegment to the left in (B) displays duplication of both dorsal-most neurons and sheath cells. (C) A hs-*numb* embryo labeled with anti Cut as well as MAb22C10. Double, parallel dendrites that indicate a duplication of the dorsal es organ in the hemisegment to the left have no Cut positive outer support cells [osc]corresponding to this es organ. Arrows indicate neuronal dendrites, open arrows mark prospero staining sheath nuclei. Box demarcated by the dotted line shows region where Cut-positive outer cells should be.

A
d
sh
B
d
d
sh
sh
C
osc
d
d

Figure 1.6. Transformation of Chordotonal Cap Cells to Neuron and Sheath Cells in hs-*numb* Embryos

(A) Diagram of lineage for ch organ cells. The neurons [n](arrows) and sheath cell nuclei [sh](open arrows) of v'ch organs from heat-shocked wild-type (B) and hs-*numb* (C,D) embryos are visualized by double labeling with MAb22C10 and anti pros. The middle hemisegment in (C) displays duplication of both neuron and sheath cell. (D) The second type of ch organ transformation observed in hs-*numb* embryos is the duplication of the neuron (arrowheads) concomitant with the loss of the pros-positive sheath cell.

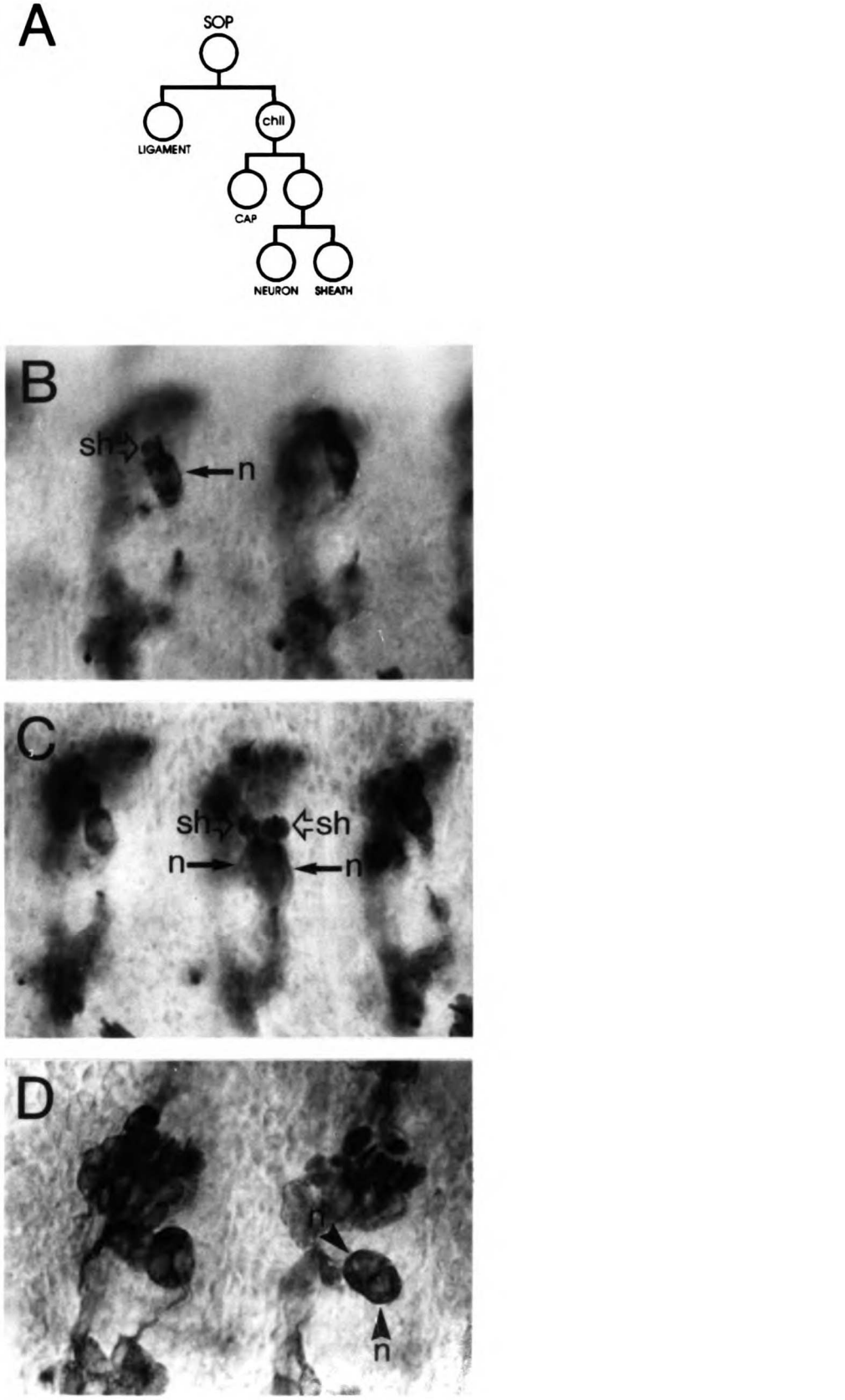

A
SOP
LIGAMENT
chII
CAP
NEURON
SHEATH
B
sh
n
C
sh
sh
n
n
D
n

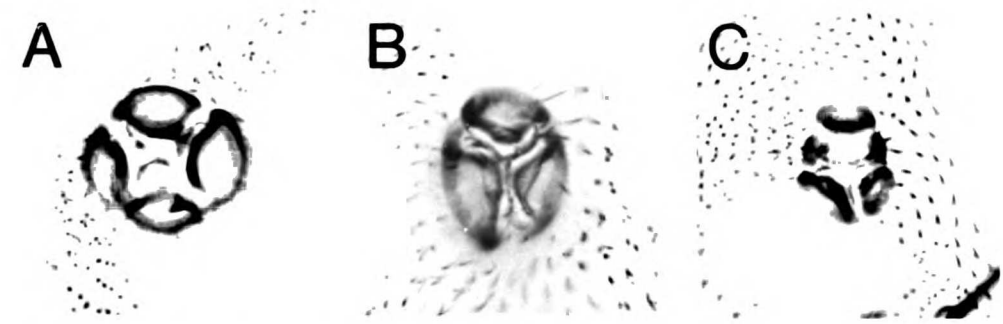
Figure 1.7. Two Distinct Transformations Resulting from hs-*numb* Induction in Adult es Organ Formation.

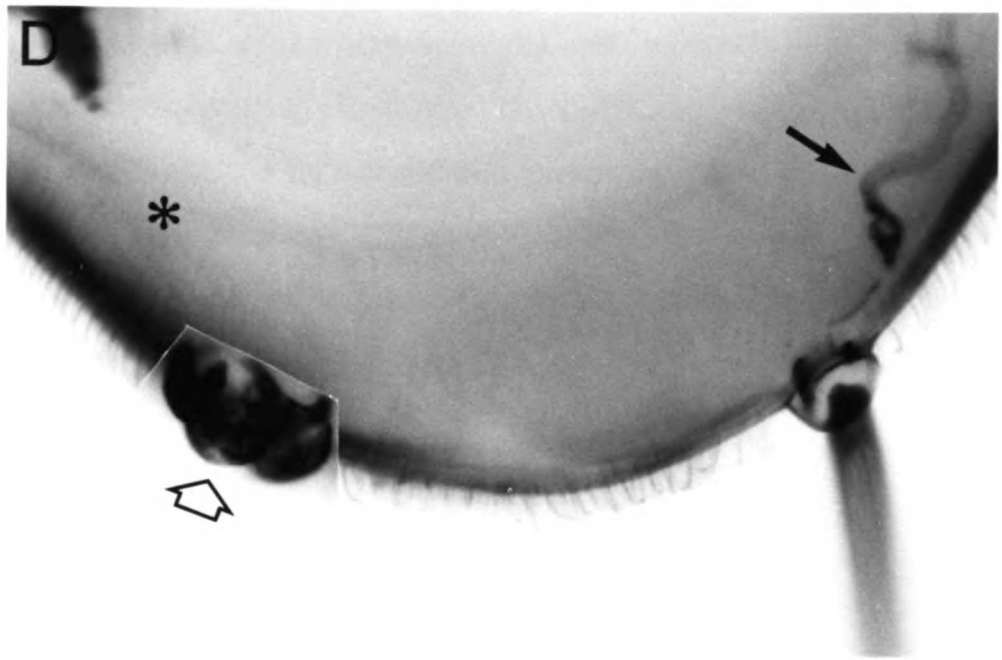
(A-C) Nota dissected from flies subjected to heat shock earlier in development. (D and E) Nota from heat shock treated pupae dissected at 40-43h APF and stained with MAb 22C10.(A and D) *yw*; Ore-R, wild type flies heat-shocked between 16-18h APF. These flies are indistinguishable from *yw*; Ore-R flies heat-shocked between 18-20h APF Arrows indicate single neurons innervating individual microchaetae. (B and E) hs-*numb*, heat-shocked between 16-18h APF, displaying the "balding" phenotype. Arrow points to duplicated neurons underneath the bald patch. (C) hs-*numb*, heat-shocked between 18-20h APF, displaying the "twinning" phenotype. (F) An anterior supra alar macrochaete from a hs-*numb* fly, heat-shocked between 12-14h APF to yield the twinning phenotype (see text), was dissected after eclosion (to enable identification of twin hairs), and labeled with MAb22C10. A neuron is associated with twin hair structure (arrow). (G,H) hs-*numb* nota often contain aberrant structures that are subcutaneous and appear to be partially transformed bristles.

A
B
C
D
E
F
G
H

Figure 1.8. Loss of numb Function in Adult Mosaic Flies: Four Socket Phenotype

(A-C) The spectrum of macrochaete abnormalities observed in mosaic nota where *numb^{3}ck^{-}* patches were induced in flies of the following genotype: *ywFlp/yw; y^{+}numb^{3}ckP[FRT]40A/P[FRT]40A*. The spotty appearance of epidermal hairs, particularly visible in focal planes in A and C, is due to the *ck-* mutation, which marks mutant patches. (D) Labeling of a mosaic patch with MAb22C10, which stains neurons in adults, shows the neuron that innervates a normal bristle (arrow), and the absence of sensory neurons (noted by asterisk) underlying a mutant bristle (open arrow). Out of the plane of focus, *ck^{-}* epidermal hairs confirm that this is a mutant patch. (E) Microchaetae within the *ck^{-}* patch designated by the dotted line produce a broader range of phenotypes: The four socket transformation (stout arrow) is seen in approximately 10% of cases; sometimes a partial hair is formed, surrounded by multiple sockets (open arrow); often essentially normal hair cells are formed whose thick appearance is due to the *ck^{-}* marker. The sockets of these microchaetae nonetheless do not form properly (curved arrow). We do not detect neurons beneath these mutant bristles (data not shown). In these experiments, the *numb^{+}/numb^{+}* twin patches were identifiable by a lack of the *y^{+}* gene, resulting in yellow bristles. These bristles have normal sensory neurons (data not shown). Control experiments in which the markers were reversed confirmed that the observed phenotype is not a result of the mutant markers (data not shown).


A
B
C


D

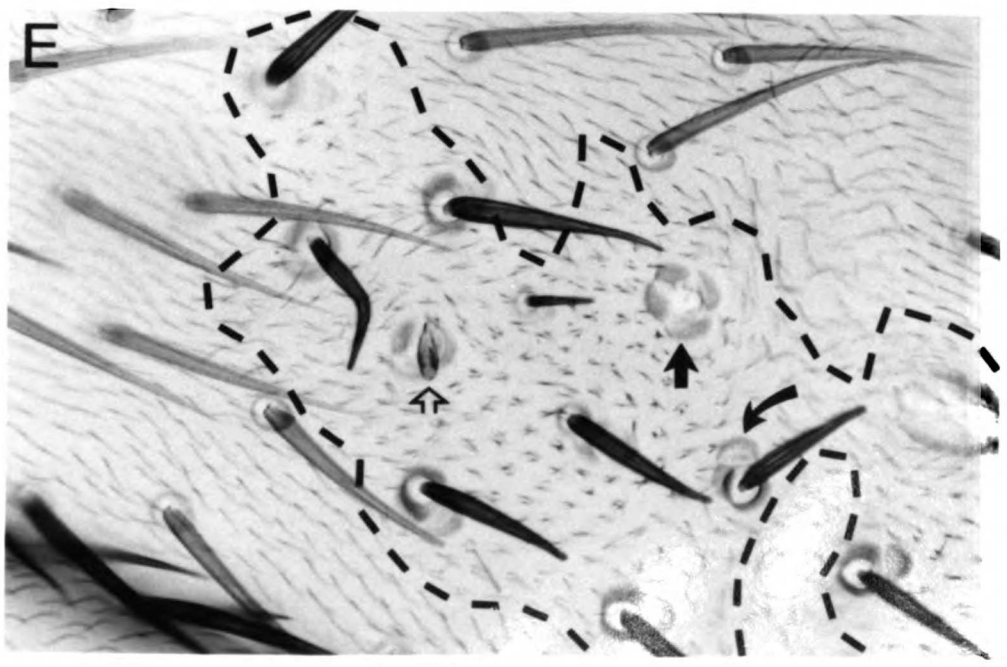

E

Figure 1.9. Proposed Lineages of Adult Misexpression and Mosaic Phenotypes

(A) The lineage giving rise to the balding phenotype. Induction of numb expression in both secondary cells results in the formation of two IIb cells. Two sets of IIb cell descendants are thus formed, giving rise to a supernumerary neuron and sheath. The daughters of the IIa cell never form, giving the outward appearance of bristle loss, or balding. (B) The lineage giving rise to the twinning phenotype. Numb induction after the division of the IIa cell transforms the socket cell into a hair cell. The IIb cell descendants appear to form normally in this case. (C) The lineage proposed for numb mutant bristles in the adult. Loss of function at both the secondary precursor stage and the four cell stage yields two IIa cells that produce only sockets (see text).

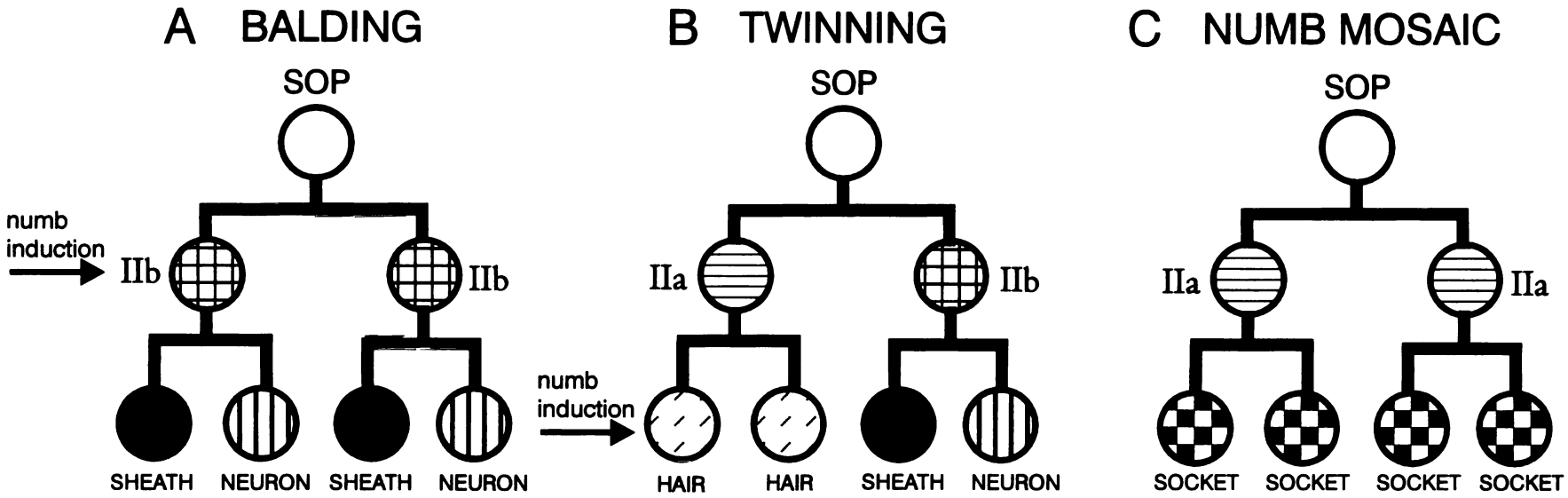
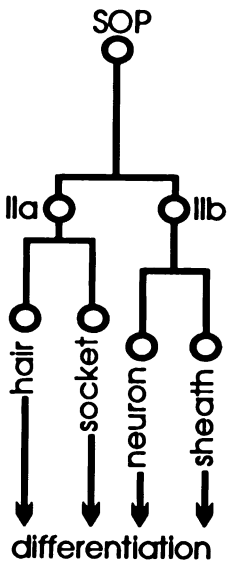

A BALDING
SOP
numb induction
IIb
IIb
SHEATH
NEURON
SHEATH
NEURON
B TWINNING
SOP
IIa
IIb
numb induction
HAIR
HAIR
SHEATH
NEURON
C NUMB MOSAIC
SOP
IIa
IIa
SOCKET
SOCKET
SOCKET
SOCKET

Table 1.1

hs-*numb* white prepupae were collected at two hour intervals for twelve hours, aged twelve hours, and heat-shocked en masse to generate samples that had been heat-shocked at the developmental times shown. Flies were examined for microchaetae loss in the region between the left and right dorso- central bristles, extending anteriorly to the head and posteriorly to the scutellum. Flies showing the bald phenotype were divided into three categories: mild, having 0-30% reduction in microchaetae; moderate, 30-70% reduction; and extreme, 70-100% reduction. The numbers displayed show both the percent of flies with extreme phenotype and the percent of flies with moderate and extreme phenotypes. Both sets of numbers indicate a peak of sensitivity to heat shock at 16-18h APF. As illustrated in the accompanying lineage diagram, this represents the two cell stage. In flies with the twin phenotype, the twinning never affected more than twenty percent of microchaetae. Thus, all flies with twinned hairs were tallied. The twinning effect appears to peak between 18-22 hAPF, corresponding to the three to four cell stages.

Table 1: Effects of hs numb induction on microchaete development



Hours APF	Time of heat shock (# of flies)	% with BALD phenotype		% with TWIN phenotype
		extreme	mod. + ext.	
12	12-14h APF (45)	0	0	0
14	14-16h APF (77)	18.2	50.7	16.9
16	16-18h APF (29)	55.2	96.6	41.4
18	18-20h APF (62)	9.7	64.5	80.1
20	20-22h APF (30)	6.7	30.0	80
22	22-24h APF (28)	0	0	0

CHAPTER 2

Dependence of numb on twins, the "B" regulatory subunit of Protein Phosphatase 2A

INTRODUCTION

Numb protein is required for the asymmetry of multiple divisions during differentiation of the es organ cells. It apparently imposes asymmetry by segregating primarily to one daughter cell in a division, promoting a particular path of differentiation in that daughter. Much remains to be understood about how numb becomes localized to one daughter and how, once distributed, numb specifies fate. One strategy to elucidating the processes involved in localization and signaling is to examine other mutants which display *numb*-like phenotypes and to determine whether numb relies on these products for localization or activity. I have taken this approach with the gene, *twins*, which encodes the B regulatory subunit of the serine/threonine Protein Phosphatase 2A [PP2A] in *Drosophila* (Uemura et al., 1993).

PP2A is a member of the superfamily of serine/threonine phosphatases which includes types 1, 2A, 2B and 2C. These types are classified by different substrate specificity, inhibitor sensitivity, and cation dependence (Cohen, 1989; Cohen et al., 1990). Initially isolated and characterized in mammalian cells, PP2A is known to exist in several oligomeric forms which contain a catalytic (C) subunit associated with either one or both regulatory subunits (A and B). Reconstitution experiments suggest that the B subunit confers substrate specificity to the phosphatase, reducing or enhancing phosphatase activity depending on the substrates used (Shenolikar and Nairn, 1991).

While several defects have been attributed to various *tws* alleles in *Drosophila*, the hypomorphic *tws* allele, *tws55*, displays a specific fate transformation in which the pIIb behaves as a pIIa cell (Shiomi et al., 1994). This transformation is similar to the effect of *numb* loss of function on the secondary precursors. Since this allele does not appear to affect the hair versus socket fate

decision, the transformation yields an ectopic hair and socket cell at the expense of the neuron and sheath cell, creating a bristle with two non-innervated hairs, each associated with a socket.

It is unclear how a serine/threonine phosphatase would contribute to this fate decision. Two possibilities are: The localization of the numb product could rely on the phosphorylation of numb or some essential localization machinery, or signaling by numb could be dependent on activation of downstream proteins via a phosphorylation/dephosphorylation mechanism. In this chapter, I summarize experiments in which I attempted to discern if localization and/or activity of numb were dependent on *twins*. I report that numb protein is not localized in CNS neuroblasts that are mutant for *twins*. It remains to be determined whether this effect is specific or secondary to a cellular defect caused by hyperactive phosphatase.

METHODS

Drosophila Stocks

Flies were raised on standard cornmeal-yeast agar medium at 25°C. Stocks of *twsP* (Uemura et al., 1993) and *tws55* (Shiomi et al., 1994) balanced over the TM6B*Tb* chromosome for easy identification of *Tb*$^{+}$*tws* homozygotes were kindly provided by T. Uemura. The hs-numb 2.4C strain used to make 2.4C/*Cyo*; *tws*/TM6[y+] is described in Rhyu et al., 1994.

Immunoblotting Analysis

Whole embryonic extracts were prepared from timed samples, run on an SDS-polyacrylamide gel, and transferred to nitrocellulose as described in Rhyu et al., 1994. Larval CNS, pupal, and adult extracts were made by homogenizing the tissue in Laemmli buffer (10 animals per 100µl buffer; 5µl of sample per lane). Blots were incubated with anti-numb primary antibody at 1:1000 diluted in TBST+5% milk. Following 2hr to 20hr incubation, blots were washed four times, 5 minutes per wash, in TBST and incubated for 1 hour with alkaline phosphatase conjugated rabbit secondary antibody at a dilution of 1:7500 in TBST + milk. Blots were washed three times, 5 minutes each with TBST and transferred to AP reaction buffer. Reactions were visualized using NBT and BCIP substrates from BioRad.

Immunofluorescence and Confocal Microscopy

CNS and optic lobes were dissected from third instar larvae. Tissues were fixed in 4% formaldehyde for 30 mins., and stained with numb as described in Rhyu et al., 1994. Chromosomes were stained by adding Hoechst dye in PBS after the final PBT wash. Samples were rinsed in PBS one time before mounting in 90% glycerol + n-propyl gallate.

Genetic Analysis

To test whether numb function relies on active twins, flies of the following genotype were generated: *yw*; hs-numb2.4C/2.4C; *tws55*/TM6*Ubxy+*. Timed *y*⁻ larvae and pupae were collected and numb was induced with a 30 minute heat shock at 39° C. Following development to pharate adult, the *yw*; 2.4C/2.4C; *tws55/tws55* flies were removed from pupal cases and examined for macrochaete phenotypes. Samples were mounted using Hoyers media. *yw*; 2.4C/2.4C; *tws55*/TM6*Ubxy*$^{+}$ and *yw*; *tws55/tws55* flies were analyzed in parallel.

RESULTS

Localization of numb is altered in twins *larval CNS neuroblasts*

Juergen Knoblich has recently correlated the localization of numb protein in neuroblasts with the stages of mitosis. He has determined that numb is distributed throughout the cell during interphase and becomes localized in the familiar crescent pattern during late prophase. The crescent is visible until the end of cytokinesis, whereupon the membrane bearing the crescent forms the membrane of one daughter cell. In collaboration with Juergen, I examined numb distribution in cells which are known to be affected by *tws* mutation.

Because the *tws55* phenotype has low penetrance in microchaetae (15%), it is extremely difficult to determine whether the *twins* mutation affects numb localization by staining of a field of microchaete SOP cells (The visible crescents may be due to residual twins activity in 85% of bristles). We therefore capitalized on a larval phenotype exhibited by the *twsP* strong loss of function allele. Originally described for the mutant, *abnormal anaphase resolution [aar]*, which is allelic to *tws*, the phenotype is characterized by abnormally condensed chromosomes during mitosis, as well as the formation of chromosomal bridges in dividing neuroblasts of the larval CNS (Gomes et al., 1993; Mayer-Jaekel et al., 1993). Since numb is asymmetrically distributed in the larval neuroblast divisions that form larval ganglion mother cells (MSR, unpublished result), the dependence of numb localization on *twins* can be determined in CNS neuroblasts which exhibit the *aar* phenotype.

While numb crescents associate with wild type brain stem neuroblasts which are in prophase, metaphase, or anaphase, no crescents can be detected in *twins* neuroblasts at the same stages (Figure 2.1). Moreover, no unlocalized numb protein can be detected in the membranes of these cells. Curiously, this effect is specific to the neuroblasts in the brain stem and medial region of the

optic lobes: The regenerating region of the optic lobes continues to show strong membrane staining and crescent localization despite displaying the *aar* condensed chromosome phenotype.

The lack of membrane associated numb staining in some *tws* neuroblasts suggests that *tws* may be required for localization of numb to the membrane, numb stability, or production of the protein. We had noticed an apparent reduction in overall numb signal in the *tws* neuroblasts, thus, we attempted to confirm whether these neuroblasts were competent to produce membrane associated numb by overexpressing numb from a heat shock promoter in *twsP* larvae. Despite the induction of ectopic numb expression, we detect no numb staining associated with the neuroblasts or epidermal cells in the CNS.

Numb protein in twins *larvae*

Using the alkaline phosphatase method of detection for western analysis, at least three forms of numb were clearly distinguishable. Figure 2.2A shows numb protein in staged embryonic extracts. Relatively faster migrating bands are detectable in extracts from 0-3 hour embryos, consistent with the size expected for the product of the 3.1 kb maternal transcript. Alternative, slower migrating bands accumulate after the start of zygotic transcription, at about 4 hours. The visualization of all of these bands in the 60kD range is specifically inhibited by the carboxy-terminal peptide to which the antibody was raised (data not shown). The observation of multiple bands presents the possibility that these bands represent differentially phosphorylated forms of the protein. To assess whether this profile of bands is altered in the tws background, I examined the numb protein recovered from *tws* mutant larval discs and brains. Figure 2.3 shows that the numb band patterns in extracts from wild type, *tws55* and *twsP* third instar larvae are indistinguishable, implying that, within our level of detection, neither the

isoform of numb nor the overall level of numb is appreciably altered in *tws* background.

Genetic Analysis

I attempted to test whether numb function relies on active twins by inducing the hs-*numb* bald phenotype in flies containing homozygous tws^{55}. The strain generated for this experiment was of the following genotype: *yw*; 2.4C/2.4C; *tws55*/TM6*Ubx*[y+]. Because the tws phenotype is most consistently seen in the anterior scutellar macrochaetae, I timed hs-*numb* induction to correspond to the first division of these SOP cells (2-4hr APF). Unfortunately, very few y^- (tws^{55}/tws^{55}) flies survived these manipulations. Combined with the moderate penetrance I observe for the tws phenotype in these macrochaetae (~50%), the low survivability made it impossible to obtain statistically significant data. This question may be more effectively asked by inducing hs-*numb* in a mosaic *tws* background.

DISCUSSION

After considering these data and weighing them with the results of others, I have realized that this work is inconclusive. In the interest of those who may pursue this line of experimentation, I will discuss the results, focusing on apparent discrepancies in the data and suggested modifications to these experiments. I will conclude with a discussion of the potential significance of phosphatase activity for numb localization.

The principal issue concerning numb dependence on *twins* is the potential generality of PP2A function. If numb localization is disrupted in *tws* mutants, it may be the indirect result of the cell's overall unhealthy state rather than a specific effect on the process that localizes numb. The chief argument against this explanation is the specificity of the transformation seen in the *tws55* mutant. This phenotype demonstrates that the cells are healthy enough to undergo complete division and to differentiate as outer support cells. Recent experiments underscore this observation: mosaic animals containing homozygous patches of a strong loss of function allele also display the double hair/double socket phenotype (Shiomi et al., personal communication). The ideal experiment, therefore, would be to observe numb localization in cells that display this phenotype. While the low penetrance of the *tws55* phenotype makes this experiment unfeasible, the availability of mosaic animals may allow visualization of numb in marked mosaic patches. By comparison, it is not clear what can be inferred from the staining pattern of numb in neuroblasts from tws^{P} larvae. Since the tws^{P} phenotype at this stage is more severe than the *numb* phenotype, this phenotype may reflect a broader role for twins at the larval stage compared to later, during bristle formation. Hence, any effect on numb localization at this stage may be the indirect result of *tws* effects on general cellular processes.

With respect to the effect of the *tws* alleles on the numb protein itself, the western analysis seems to indicate that no remarkable alteration occurs in a *tws* background. Though this appears to support the proposal that numb is produced but fails to localize to the membrane in *twsP* larvae, the fact that extracts were made from all imaginal disc tissues instead of exclusively those that fail to localize numb weakens this conclusion. We have observed that the extent of disruption of the numb staining pattern in *twsP* larvae varies, even among the different tissues of the larval CNS. Thus, extracts made from all imaginal discs may obscure any effect on numb product in the brain stem. The more compelling experiment is to analyze numb level and isoforms in extracts of dissected brain stems from *twins* larvae.

It should also be noted that another investigator observes differences in the numb bands in extracts from *twsP* and *tws55* larvae, compared to wild type. Shiomi et al. observe a shift to the fastest migrating isoform of numb (personal communication). They interpret this altered mobility as a reflection of a shift of the numb protein population to a predominantly unphosphorylated form, suggesting hyperactivity of the phosphatase. We have been unable as yet to resolve the differences between our experimental techniques. To a first approximation, extracts are prepared from the same tissues in the same manner, and similar SDS-PAGE conditions are employed. If the Shiomi observation can be upheld, interesting models could be fashioned for the dependence of numb stability, localization or activity on its phosphorylation state.

Alternatively, the phosphatase may act on another substrate which is necessary for localization of numb. The phenotype of the yeast homolog of twins, *cdc55*, provides a potential connection between phosphatase activity and behavior of microfilaments. The budding defect in cdc55 mutants is similar to the phenotype observed in mutants defective in the 10nm filaments, offering the

possibility that *twins* may regulate the polymerization of these filaments (Healy et al., 1991). Such regulation of cytoskeletal rearrangement may exemplify the type of structural reorganization on which numb localization may rely.

Figure 2.1. Numb expression in *twins* mutant larval neuroblasts

Numb protein expression in CNS neuroblasts from wild-type (A) and tws^P (C) larvae. Arrow points to the crescent pattern of numb localization. Lack of any detectable numb staining in (C), whether asymmetric or uniform, typifies our observations. (B and D) Double staining of same samples with Hoechst dye indicates these cells are in metaphase. (B) shows the wild-type cell containing condensed chromosomes aligned at the metaphase plate (indicated by arrow). The metaphase plate in (D) illustrates the hypercondensed chromosome phenotype of the tws^P neuroblast. Note that in tws^P larvae, some abnormal, apparently necrotic cells are detected which have intense, artifactual numb staining around their periphery (data not shown). These cells are clearly not neuroblasts.

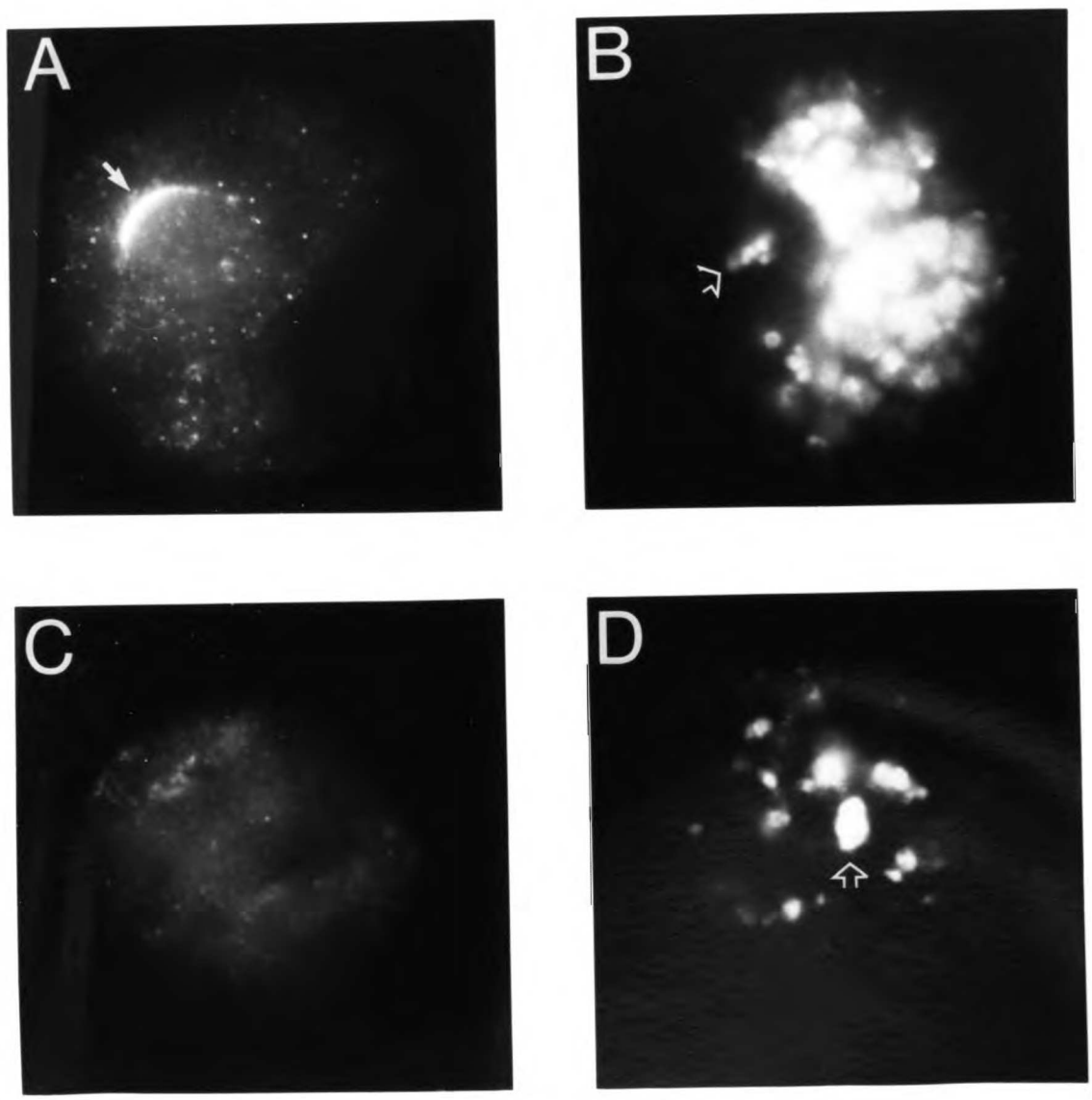


Figure 2.1

Figure 2.2. Numb protein from staged embryonic extracts

Western blot analysis of extracts from 0-3 hr (lane 1), 3-6 hr (lane 2), 6-9 hr (lane 3), 9-12 hr (lane 4) and 12-15 hr (lane 5) embryos. Bands in the 60-65 KD range are specifically inhibited by 100nM C-terminal peptide to which 1053 numb antibody was generated, but are not inhibited by same concentration of N-terminal 30 amino acid peptide (data not shown). Marked size differences in numb from 0-3 hr embryos may result solely from difference in size of maternal transcript (3.1Kb verses zygotic 3.5 Kb), or may result from differential modification of the protein.

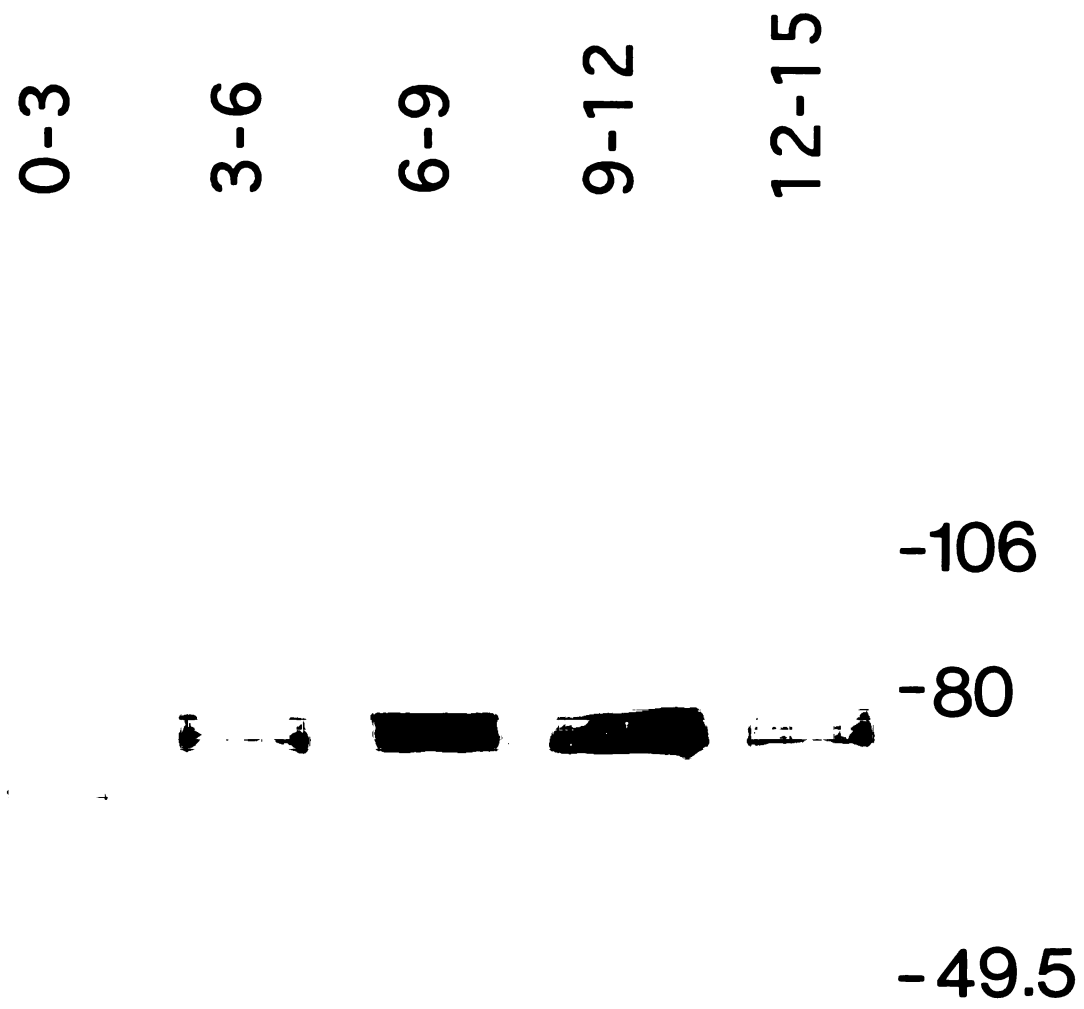


Figure 2.2 Numb Protein from Staged Embryonic Extracts

Figure 2.3. Numb protein from *Ore-R*, *twins*^{P}, and *twins*^{55} imaginal disc extracts

Comparison of extracts prepared from imaginal discs and CNS (including optic lobes) of *twins*^{P} (lane 1), *twins*^{55} (lane 3), and *Ore-R* (lanes 2 and 4) third instar larvae. The bands within the cluster indicated by brackets are specifically inhibited by addition of the C-terminal numb peptide to primary antibody incubations (data not shown), and are therefore believed to represent variously migrating forms of numb. The mobility of this set of bands is not significantly altered in either *twins* mutant.

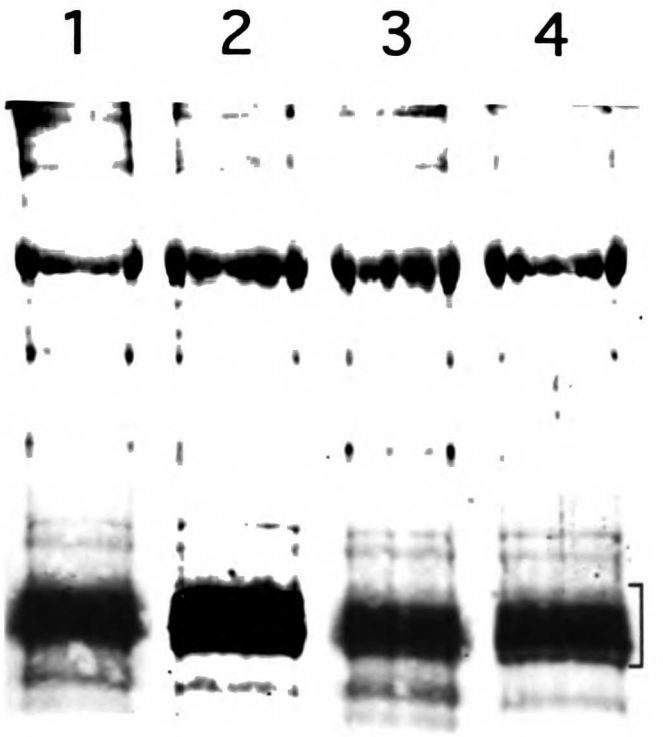


Figure 2.3 Numb Protein from *Ore*^{R}, *tws*^{P}, *tws*^{55} Imaginal Disc Extracts

CHAPTER 3

numb and *Notch*: How does the intrinsic signal converge with the extrinsic signal?

INTRODUCTION

Although the asymmetric distribution of numb constitutes an intrinsic difference between daughters of the SOP cell, this result does not preclude the role of cell-cell signaling in the determination of these cell fates. On the contrary, the cell fate defects observed in *N*, *Dl*, *neu*, *Su(H)* and *H* mutants suggest that the process of lateral inhibition may be involved in this fate specification. Since cell fate phenotypes can be observed in mutants for either *numb* or the genes involved in extrinsic signaling, it appears that both extrinsic and intrinsic signals are necessary for proper fate assignments. An important issue, then, is how the the numb signal and the signals that mediate lateral inhibition interface to determine cell fates.

Despite the identification of many genes involved in SOP cell selection via lateral inhibition, the mechanism of this inhibition has only begun to be understood. Several lines of evidence, including adhesion studies in cultured cells (Fehon et al., 1990), investigations of cell autonomy in mosaic animals (de Celis et al., 1991a; Heitzler and Simpson, 1991a; Hoppe and Greenspan, 1986), and epistasis tests (de la Concha et al., 1988), strongly support the model that Delta in the SOP cell sends a signal that is received by Notch on neighboring cells in the proneural cluster. This activation of Notch is thought to trigger a signal transduction pathway that leads to repression of the *Achaete-Scute* genes and, eventually, to epidermal fate in the surrounding cells (Artavanis-Tsakonas and Simpson, 1991; Ghysen et al., 1993a).

The recent report by Fortini et al. (1994) proposes that the Notch signal is mediated through Su(H), which, upon Notch activation, translocates to the nucleus, where it may act as a transcriptional repressor (Fortini and Artavanis-Tsakonas, 1994). This model is consistent with the observation that the domain of scute expression is expanded in Su(H) mutant wing discs (Schweisguth and

Posakony, 1992). Hairless is observed to antagonize DNA binding by Su(H) (Brou et al., 1994). It is unclear where the other neurogenic genes fit in this model. Although this proposed interplay of N, Dl, Su(H) and H in SOP selection has not been strictly proven for the role of these genes in the es cell fate decisions, the phenotypes observed in es organs are consistent with the model. Therefore, it serves as a useful reference for asking how numb interacts with this signaling pathway during specification of the IIa and IIb cells.

In the limited investigation described in this chapter, I attempt to order *numb* and *Notch* in an epistatic pathway. Given the experimental difficulties posed by the multiple roles of Notch during SOP selection and differentiation, I examined the effects of heat shock numb expression in a fly that expresses activated Notch [NotchΔ] in descendants of the SOP cell. I report that the NotchΔ phenotype is epistatic to heat shock numb, implying that numb does not act downstream of the Notch protein.

METHODS

Drosophila Stocks

Flies were raised on standard cornmeal-yeast agar medium at 25°C. The UAS-NotchΔB2A3 strain was generated in the lab using an N-terminal Notch deletion constructed by E. Giniger. The GAL4 insertion used in this study, 109(2)68, appears to be located in scabrous gene (S. Shepherd) and was generated by L. Luo and S. Ralls in the lab. Two hs-numb chromosomes, 2.4C (on chromosome II) and 11.1 (on chromosome III), were employed. The generation of these independent hs-numb insertions is described in Rhyu et al., 1994.

Genetic Analysis

Stocks of the following genotype were generated: 109(2)68/*CyO*; 11.1/TM3*Sb* and 2.4C/2.4C; B2A3/*TM3Sb*. Males of the first genotype were mated to females of the second genotype and progeny larvae were collected and aged to 18-20 hours. Numb expression was induced in these larvae with a 30 minute heat shock at 39°C. Nota from newly emerged adults were dissected and mounted in Hoyer's media for analysis.

Flies of the genotype 2.4C/109(2)68; B2A3/11.1 were distinguished by Sb^+ and the characteristic double socket phenotype of 109(2)68; B2A3 containing flies. 2.4C/109(2)68; 11.1/*TM3Sb* flies were analyzed in parallel as a control.

RESULTS

Constitutively active Notch displays antineurogenic phenotype when expressed in es organ cells

Various groups have demonstrated that a truncation of the extracellular N-terminal region of Notch results in a protein which is constitutively active. Ed Giniger in our lab has constructed a similar version of the Notch protein expressed from the UAS promoter. When this product is induced in strains that express GAL4 in proneural clusters and es organ cells, three distinct cell fate phenotypes are observed in macrochaetae (Figure 3.1): A loss of the es organ, a two socket organ that lacks a hair cell, and a four socket organ. All three phenotypes can be interpreted as opposite to the effects observed for *Notch* loss of function (see Table 1). Thus we conclude that the truncation encodes an activated Notch [*NotchΔ*]. The variability in the phenotypes observed for macrochaetae may reflect the nonuniform expression of GAL4 among different SOP cells and their descendants.

In the *NotchΔ* insertion line, B2A3, microchaetae exhibit a moderate transformation phenotype (Figure 3.3A). Although the bristles do not differentiate normally, they appear in normal positions at a density comparable to wild-type microchaetae, indicating that SOP cell selection is not impaired. Some microchaetae comprised of multiple sockets can be seen, but the predominant transformation in these small bristles is the two-socket phenotype (Figure 3.2A). Given that these double socket structures appear to be normally innervated (Figure 3.2B), I interpret this phenotype to be a transformation of the hair into a socket cell. Because this phenotype is opposite to the twin hair phenotype observed when numb expression is induced at 18-20 hours, it presents an opportunity to isolate the action of these proteins in one fate decision (that

between the hair and socket), to determine whether the hs-*numb* or the *NotchΔ* phenotype is epistatic in this decision.

Activated Notch is epistatic to heat-shock numb

Two copies of hs-*numb* were introduced into a background where *NotchΔ* was expressed in the 109(2)68 pattern. When numb is induced at 18-20 hours after puparium formation in control flies that lack the Notch construct, an average of 13% of the microchaetae organs that lie in the region between the left and right dorsocentral macrochaetae display the twinning phenotype described earlier (Figure 3.3B, Rhyu et al., 1994). Analysis of this limited area was chosen because the administered heat shock produces twin hairs predominantly in this region. By comparison, only 1% of organs in this region display twinned hairs in the background of *NotchΔ* (see Table 3.1). Thus, hs-*numb* cannot override the constitutive activity of *NotchΔ*, suggesting that *numb* is not downstream of *Notch* in the signaling pathway.

A second finding is that flies expressing hs-*numb;NotchΔ* appear to have a significantly greater number of total organs than hs-*numb* alone. The reduced bristle count in hs-*numb* is due to a transformation from IIa cell to IIb cell (Rhyu et al., 1994). It appears that this "balding" phenotype is also suppressed in the *NotchΔ* background.

DISCUSSION

Activated Notch yields cell transformations opposite to loss of Notch function

Although the phenotypes observed when expressing activated Notch in the proneural cluster, the SOP and its descendents have not been examined in detail, they appear to be consistent with the expected antineurogenic effect. The phenotype where no macrochaete organ is visible may be a reflection of a failure to form an SOP cell due to unmitigated mutual inhibition among the cells in the cluster. The differentiation of two sockets and no hair illustrates the potential function of *Notch* in the hair/socket decision, a role which is predicted based on the stepwise model of fate acquisition described earlier. These organs appear to have normal neurons. The third phenotype observed elaborates four sockets, suggesting that two IIa cells have formed, and each has given rise to two socket cells. All three phenotypes appear to cause a cell fate transformation in the direction opposite to the loss of function phenotype, indicating that this N-terminal truncation of Notch is constitutively active. Other activated forms of Notch which contain only the cytoplasmic domain have been observed to translocate to the nucleus (Lieber et al., 1993; Rebay et al., 1993; Struhl et al., 1993). Because this construct contains the transmembrane domain, it strongly suggests that nuclear targetting of the truncated protein is not required for constitutive activity. A mechanism by which the cytoplasmic domain of Notch is clipped off for transit to the nucleus has not been eliminated.

How do numb and Notch interact?

The prevailing lateral inhibition model states that Notch is the receptor for an inhibitory signal sent by a neighboring cell via the ligand Delta. Thus, Notch transduces this inhibitory signal in the receiving cell. The phenotypes of *numb* and *Notch* indicate that numb is required for IIb and hair fate while Notch is

required for IIa and socket fate. It is unknown what the default fates are in these divisions, but a reasonable interpretation is that the IIb cell prevents its sister from differentiating as IIb via the Dl/Notch pathway. The inhibited cell would then differentiate as IIa. Similarly, the hair cell could inhibit its sister from differentiating as hair, causing it to adopt the alternative, socket fate. My analysis of activated Notch and hs-numb was an attempt to understand where numb may fit in this pathway. I tested this interaction in the hair versus socket fate decision, because *NotchΔ* affects this division consistently in microchaetae. However, any relationship that is uncovered between numb and Notch in the hair/socket decision is expected to be the same for the IIa/IIb decision, and possibly for the sheath/neuron decision. The possibilities for *numb* and *Notch* interaction are diagrammed in figure 3.4.

A model for N acting upstream of numb is difficult to imagine, though it is formally possible (Figure 3.4A). If numb is partitioned to the IIb/hair/neuron cell, it could inhibit Notch (Figure 3.4B) or inhibit a component that is activated by Notch (Figure 3.4C). If the IIa/socket/sheath cell inherits numb, numb could induce IIb/hair/neuron fate in its sister cell by activating Notch in the IIa/socket/sheath cell (Figure 3.4D; the rationale of this model is explained below).

Perhaps the most compelling argument against the model where Notch acts on numb is the fact that numb is partitioned to only one of two daughter cells. It makes little logical sense to inhibit the protein in the only cell that acquires it. The rare occurence of twin hairs in hs-*numb*; *NotchΔ* flies supports the model that *numb* acts upstream or parallel to *Notch* but not downstream. A serious caveat to this interpretation is that the activated Notch may be so hyperactive as to succeed in inhibiting even overexpressed levels of numb.

Alternatively, *NotchΔ* may use an entirely new pathway, in which case this construct would be useless for epistasis studies.

A model wherein numb acts upstream to Notch can be combined with current understanding of *mutual* inhibition to produce a working model of how extrinsic and intrinsic information may coordinate to produce proper fates (Figure 3.5). One version of this model has been forwarded in a recent review by Jan and Jan (in press). If numb acts upstream of Notch, it may actually impinge on the events that precede lateral inhibition. Mutual inhibition describes the intercellular interactions which occur before the SOP is selected (Figure 3.5A)(Heitzler and Simpson, 1991a). These interactions take place among roughly equivalent cells in the proneural cluster via Notch and Delta. The hallmark of mutual inhibition is the feedback regulation which allows cells that receive less inhibitory signal through Notch to up-regulate their own inhibitory signal, Dl, while cells that receive more inhibitory signal decrease their levels of Dl. In this manner, a small initial difference that arises from random fluctuations in Dl signaling or N receptor activity will be amplified through the feedback loop until the cell which has highest Dl activity becomes the SOP cell. This SOP then continues to laterally inhibit its neighbors.

By analogy, the daughters of the SOP cell may begin by mutally inhibiting the IIb fate (Figure 3.5B). An amplification of a slight difference in Notch or Dl activity would lead to the selection of one IIb cell, which would have increased Dl and decreased N activity. This cell would continue to inhibit IIb fate in its sister, which would lose its ability to inhibit its neighbor. Considering that these cells divide and differentiate within a relatively brief span of time, one might imagine that the feedback loop may not have sufficient time to select a dominant IIb cell consistently. In this case, the role of numb may be to speed this selection by substantially altering the activity of Notch in one cell. If inherited by the IIb cell,

numb could inhibit Notch activity in this cell and would gain an immediate advantage over its sister to assume the IIb fate. This IIb cell would continue to inhibit its sister cell from this course, shunting it to IIa fate (Figure 3.5C). If inherited by the IIa cell, numb could activate Notch in this cell, causing decreased levels of Dl and, in effect, inducing the neighboring cell to increase its Dl levels and differentiate as IIb (Figure 3.5D). This second possibility is less direct but nonetheless possible.

In the absence of numb, slight differences may arise between the sister cells, but time may not permit these differences to be amplified sufficiently to favor one cell. Mutual inhibition would persist between the two cells, causing both cells to differentiate as IIa, as is observed in numb mutants. At some frequency, the difference may have the opportunity to amplify itself, in which case the organ would differentiate normally. This model would explain the observation of normal bristles in large *numb*$^-$ clones.

Although a genetic test of this proposed relationship between *numb* and *Notch* will be difficult, it may be possible if the role of these genes in one asymmetric division of the es organ, perhaps the hair/socket division, can be isolated. The experiment would require generation of small *Notch* clones in a background where one copy of Notch$^+$ is provided. The mitotic recombination would have to occur at the division of the IIa cell, resulting in one daughter that expresses only the single background copy of Notch and one that expresses three copies. The mutual inhibition model would predict that the cell with less Notch would always differentiate as a hair. In the background of the viable numb^4 allele, which exhibits double socket phenotypes, one would expect this effect to be more pronounced.

Does numb inhibit an inhibition to specify fate?

The possibility that numb may act in a linear pathway through Notch raises the interesting point of whether numb itself is required to activate genes leading to IIb or hair cell fates, or if numb is merely required to alleviate a Notch mediated signal which prevents an existent IIb or hair program from being carried out. Other examples of "derepression" mechanisms have been described (Hemmati-Brivanlou et al., 1994b; Hemmati-Brivanlou and Melton, 1994a; Waring and Kenyon, 1991; Waring et al., 1992). During neural tissue formation in Xenopus, activin inhibits neuralization, and secretion of follistatin by neighboring mesodermal cells alleviates the inhibition. Oocytes expressing a dominant-negative form of activin can produce neural tissues in the absence of mesodermal signals (Hemmati-Brivanlou et al., 1994b; Hemmati-Brivanlou and Melton, 1994a).

The fact that *numb*$^-$ clones in the adult contain some completely normal es organs lends support to the idea that numb is not required for activating particular cell fates. To test whether numb acts strictly through inhibiting Notch, a double mutant that is null for *Notch* and *numb* must be examined in a true epistasis experiment. Unfortunately, this experiment may be difficult to analyze due to the role of both genes in multiple steps of differentiation. If numb's role is to inhibit Notch in one daughter cell per es division, loss of both genes would be expected to give rise to two IIb cells and four neurons per organ. If numb and Notch act in parallel pathways, intermediate phenotypes may be expected.

Figure 3.1. Bristle phenotypes of activated Notch

Various phenotypes observed for macrochaetae in flies of the following genotype: 109(2)68/Cyo; NotchΔB2A3/+. (A) Scutellum from fly expressing activated Notch lacks left posterior scutellar bristles (asterisks mark sites of expected bristles). In addition, the right anterior scutellar bristle (open arrow) appears to form four sockets. (B) Two dorsocentral macrochaetae display two socket phenotypes which resemble Hairless-like hair to socket transformations. Another four socket scutellar macrochaeta is visible on this notum (open arrow). These multiple socket phenotypes are reminiscent of the transformations detected in *numb*$^{-}$ mosaic patches.

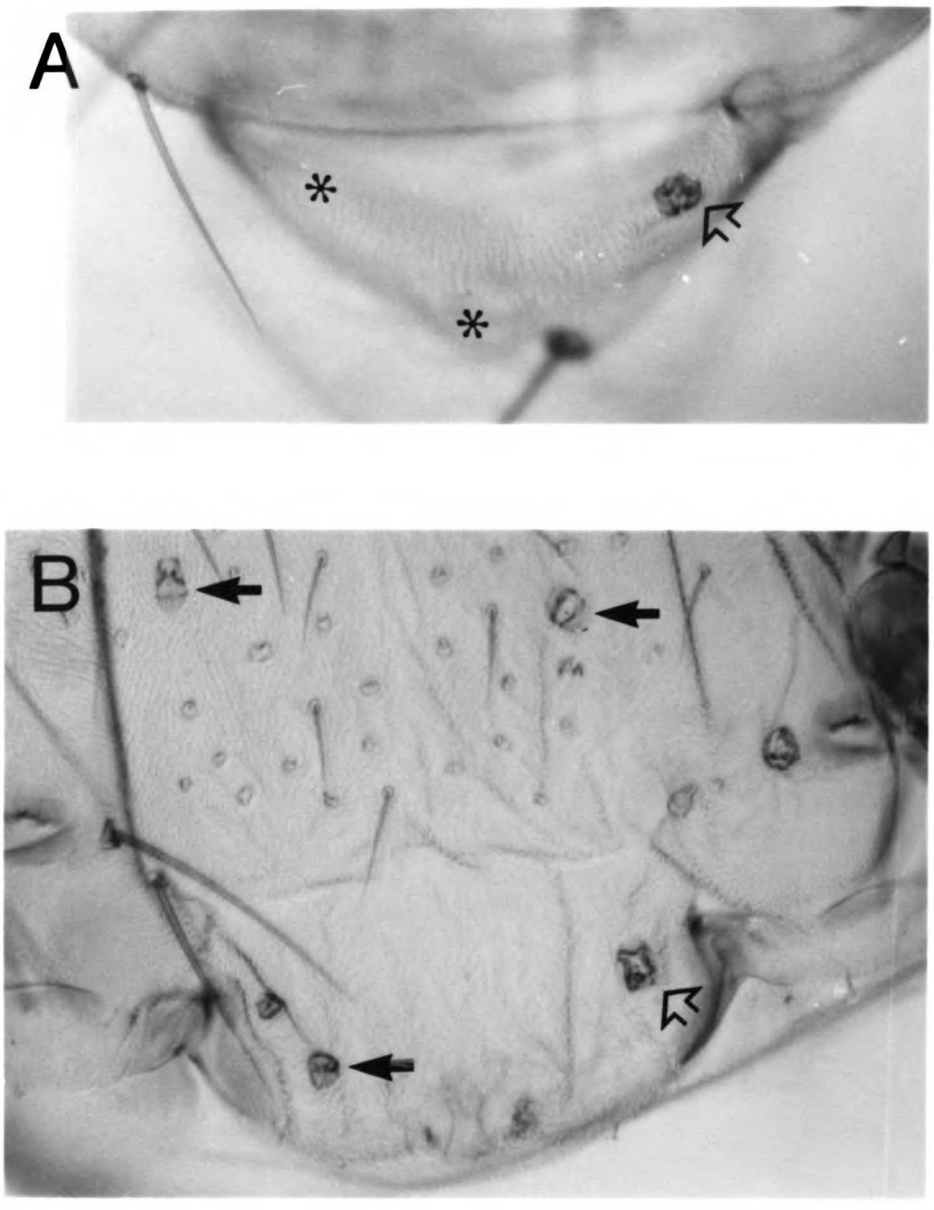


Figure 3.1

Figure 3.2. The microchaetae two-socket phenotype

(A) A section of a notum from a fly expressing activated Notch highlights two microchaetae displaying a phenotype similar to the two-socket phenotype observed in macrochaetae (arrows). (B) Staining with anti-22C10, which recognizes cytoplasmic antigens in the neuron, reveals neurons differentiate appropriately in both organs.

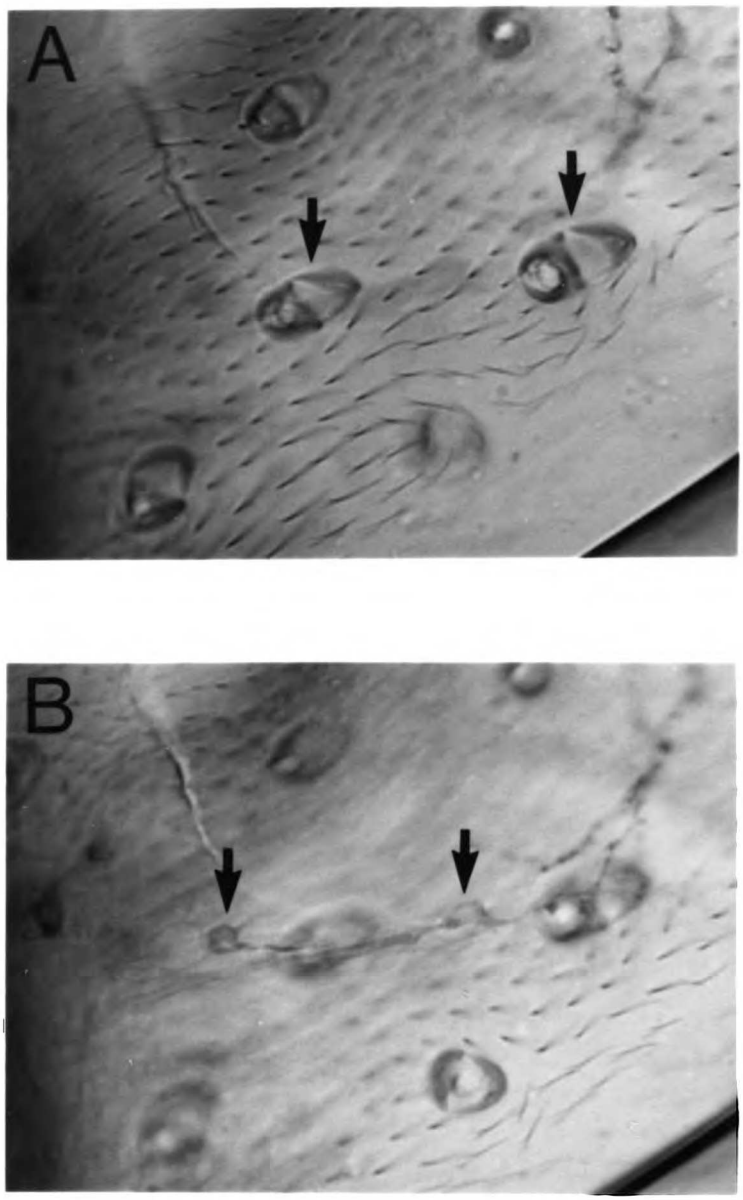


Figure 3.2

Figure 3.3. Effects of hs-*numb*; *NotchΔ* in hair vs. socket fate

(A) Microchaetae on notum of a typical 109(2)68/Cyo; NotchΔB2A3/+ fly. Many bristles show no phenotype. Arrows indicate organs that display the predominant two-socket phenotype. (B) A representative notum from a 109(2)68/2.4C; 11.1/TM3*Sb* fly that was subjected to heat shock at 18-20 hr. Bristles with the socket to hair transformation are indicated. Thick, stout appearance of bristles is due to the *Sb* marker. (C) A representative notum from a 109(2)68/2.4C; 11.1/NotchΔB2A3 fly that was subjected to a heat shock at 18-20 hr. Organs resemble microchaetae in (A).

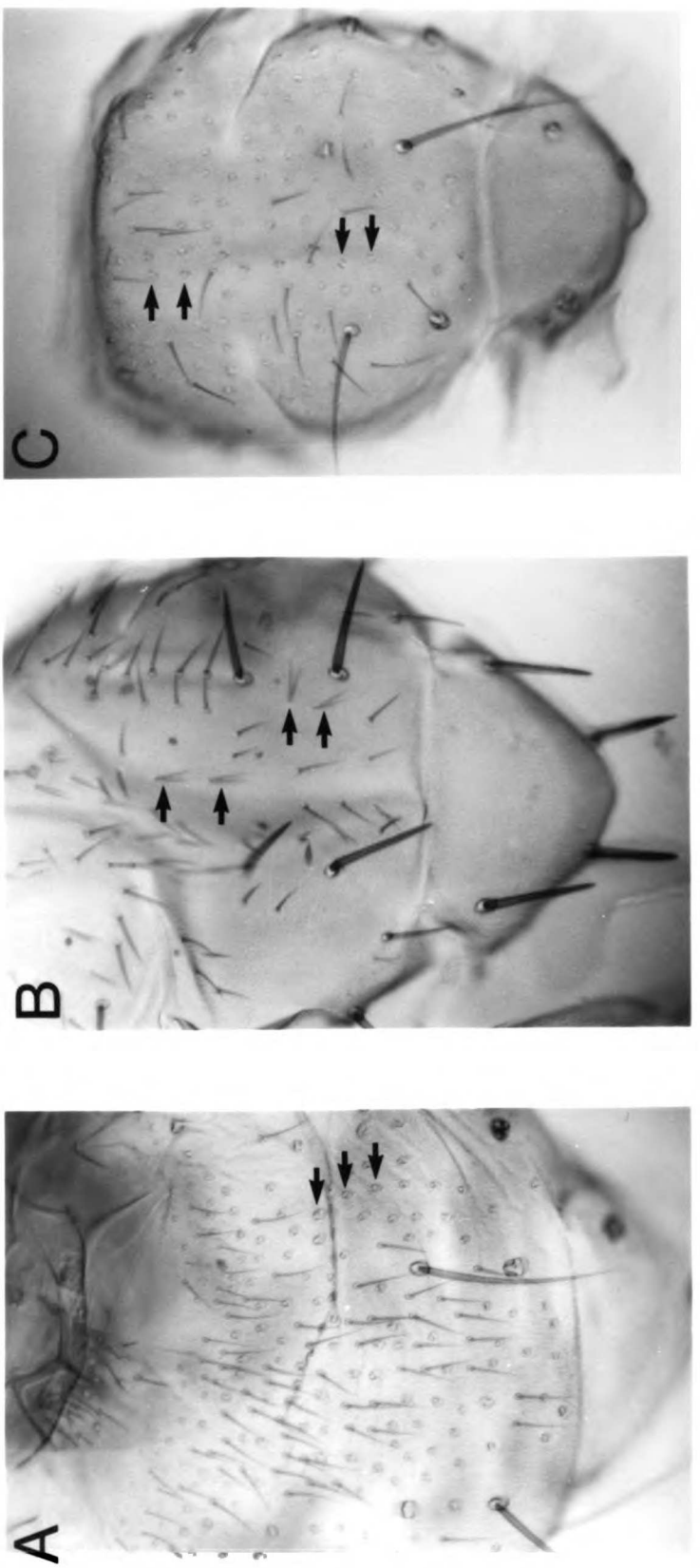


Figure 3.3

Figure 3.4. Possible Order of *Notch* and *numb* action

(A) Notch acts upstream of numb; reciprocal phenotypes of these mutants suggest this would be an inhibitory interaction. (B) Numb inhibits Notch in a linear pathway; (C) numb inhibits a putative responder to Notch activation (labeled X). Possibilites (B) and (C) demand that numb act in the IIb cell. (D) Reciprocal mutant phenotypes can also be explained by a model where numb activates Notch. This option depends on numb action in the IIa cell.

Figure 3.4 Possible Order of *Notch* and *numb* Action

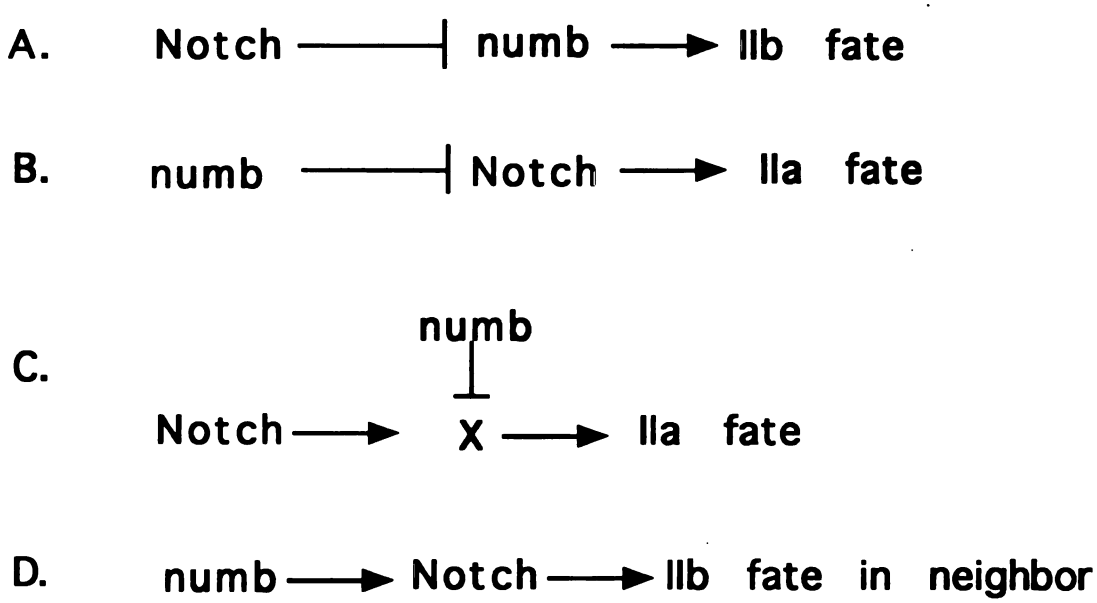


Figure 3.5. Models: Numb biases mutual inhibition

(A) The basic model of mutual inhibition, as it is postulated to occur in the selection of the SOP cell from a field of epidermal cells. Although only one future epidermal cell is represented, many would participate in the interaction. The cells would begin with equal levels of Notch and Delta activity that cause mutual inhibition of the SOP fate (1). A small difference in these activities between the cells may result from random fluctuations (2). Feedback mechanisms within each cell would result in the magnification of the original difference, resulting in one cell with elevated Delta levels and low Notch levels, and the other cell with high Notch and low Delta. The cell expressing high Delta would retain the ability to inhibit SOP fate in its neighbors, while the other cell would lose this ability, enabling the cell with high Delta to differentiate as SOP (3) (For simplicity, changes in N and Dl activity are depicted by altering the number of molecules on the cell surface. In fact, activity fluctuations probably result from post translational modifications, not recycling of molecules at the cell membrane).

(B) Applied to IIb, hair or neuron formation, this mechanism may not be efficient enough to consistently select a cell to express high Delta activity. This would result in undeterred mutual inhibition and to differentiation of both cells to the non-IIb, hair or neuron fate.

(C) Numb distribution to the IIb cell may inhibit Notch activity specifically in this cell (Numb is represented by squares containing stripes). This inequality will be amplified by the feedback system, favoring the rapid formation of a cell with high Delta activity. Lacking an inhibitory signal from its neighbor, this cell would presumably differentiate as IIb, hair or neuron.

(D) Numb could also be distributed to the IIa cell and activate Notch activity in this cell. This increased Notch activity would cause decreased Dl in this cell,

Figure 3.5A Model of Mutual Inhibition in SOP Cell Selection

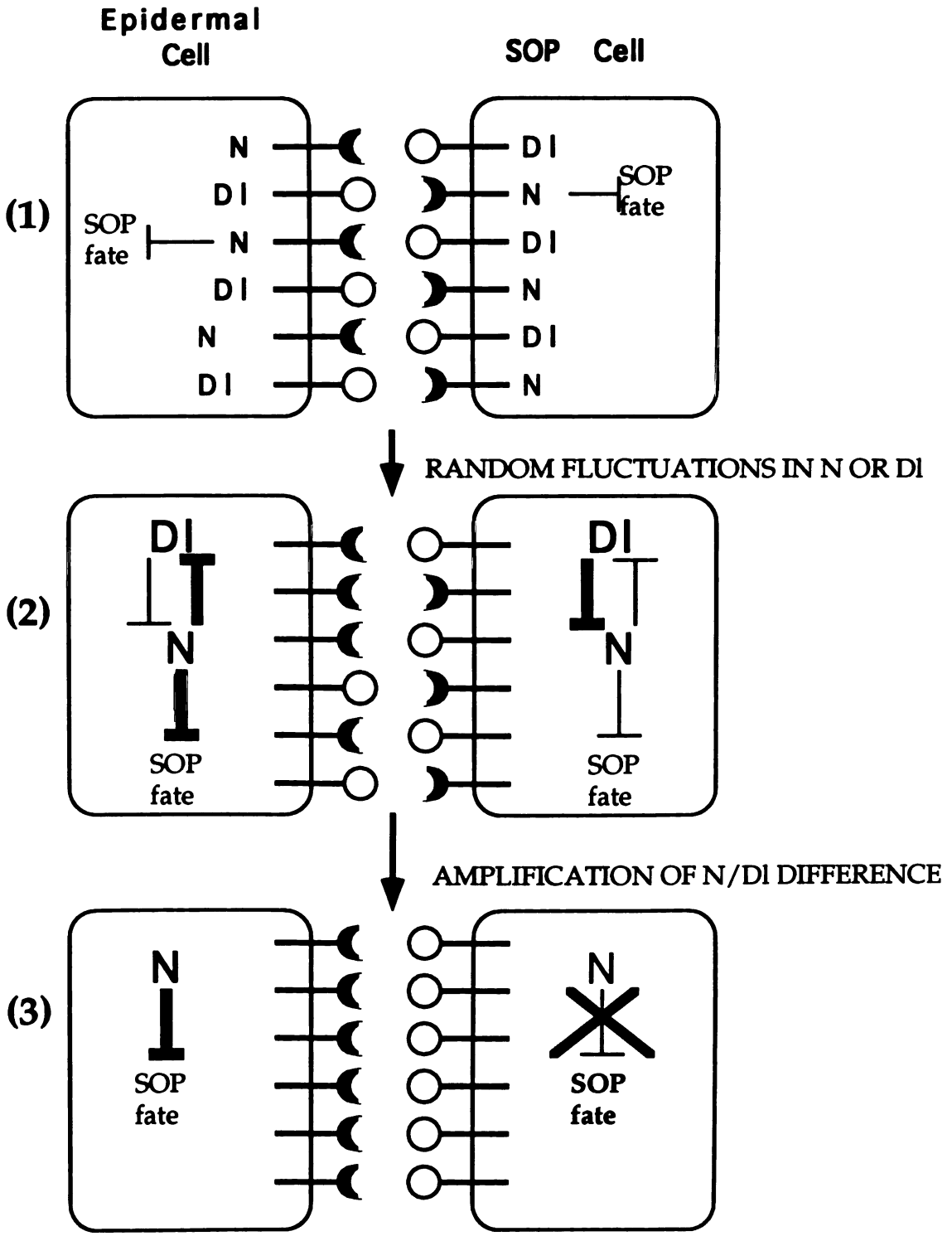


Figure 3.5B Model of Mutual Inhibition in es Asymmetric Cell Divisions

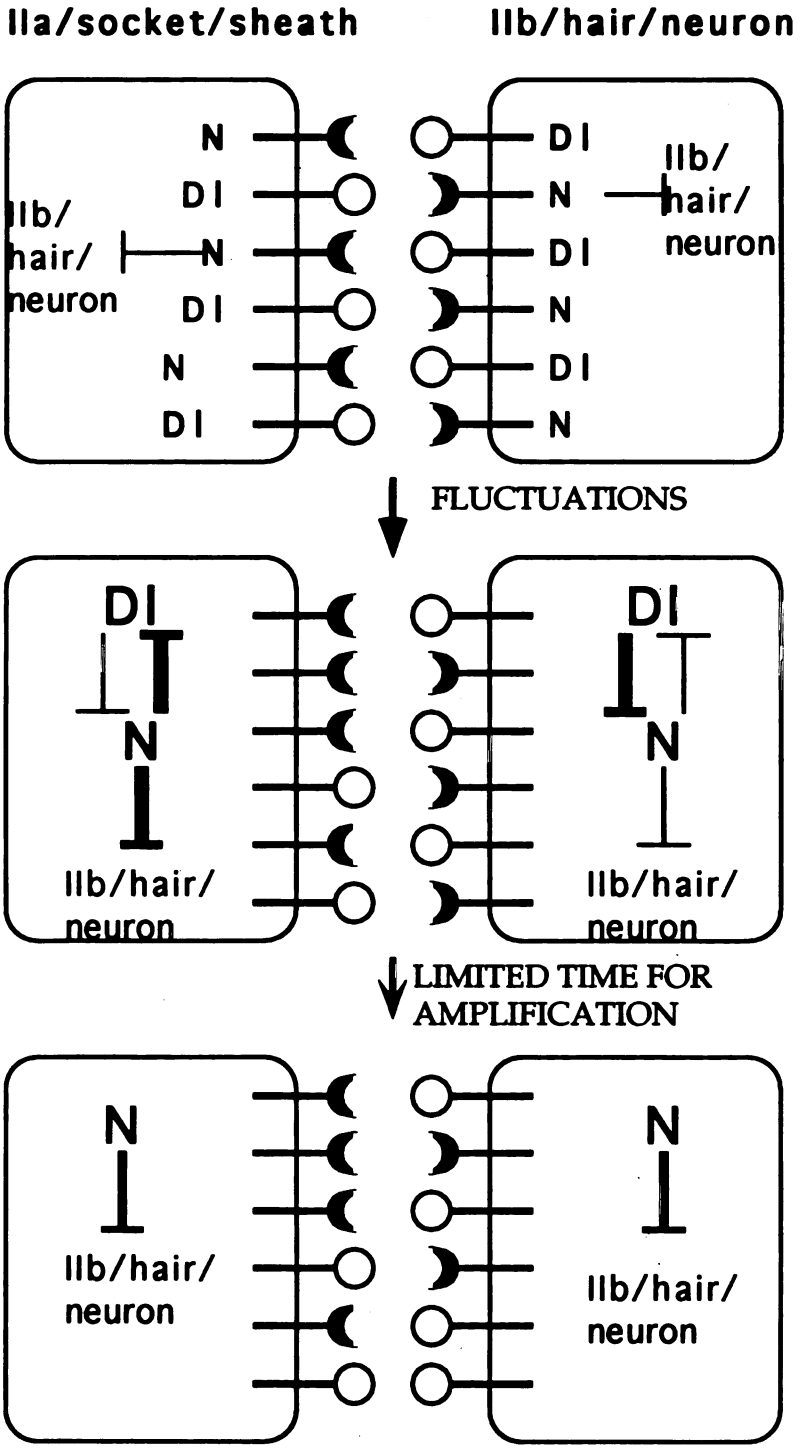


Figure 3.5C MODEL I: Numb Inhibits Notch in the IIb Cell

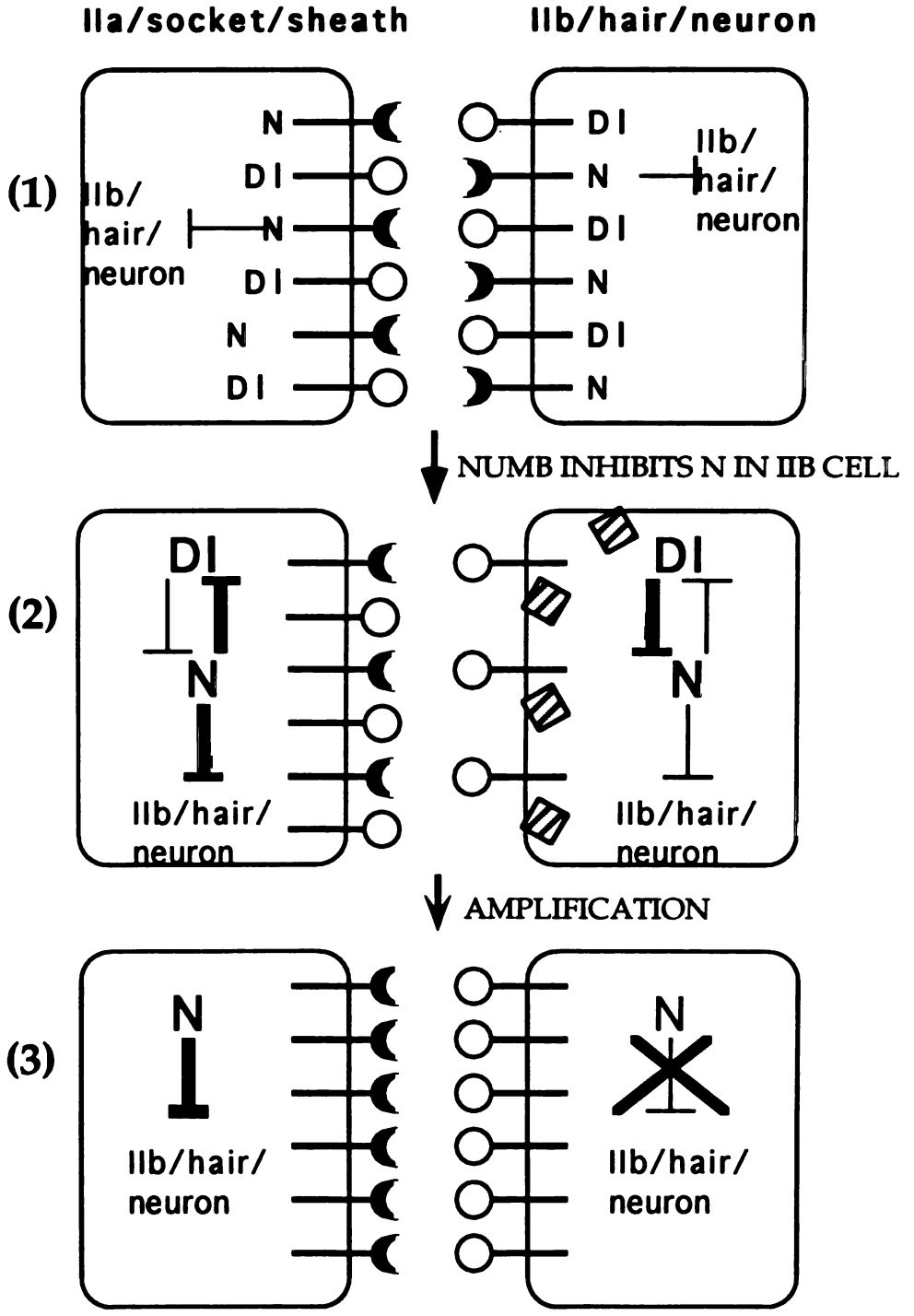


Figure 3.5D MODEL II: Numb Activates Notch in the IIa Cell

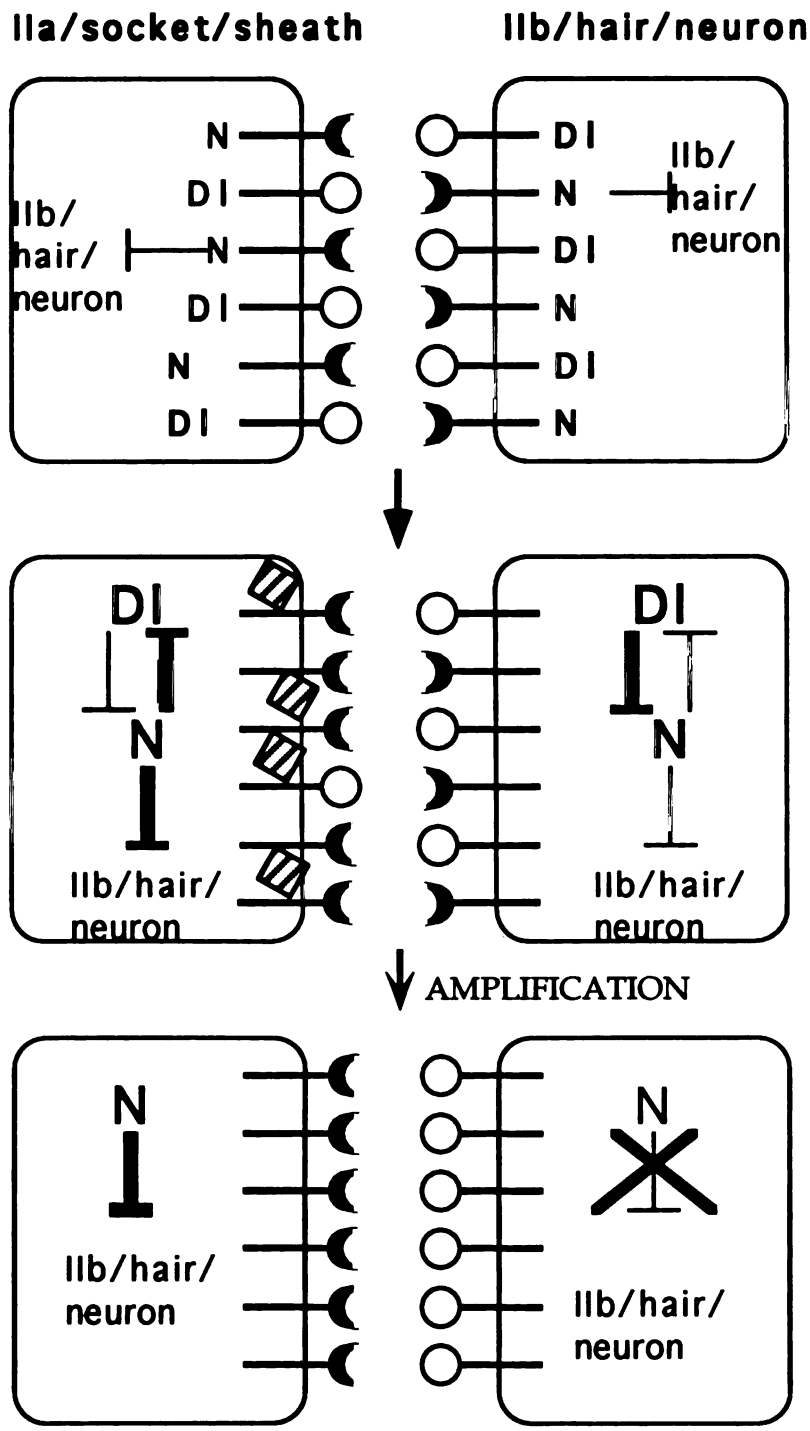


Table 3.1. Microchaete phenotypes in hs-*numb*; *NotchΔ* nota

Data are compiled from 20 nota of the genotype, 109(2)68/2.4C; 11.1/TM3Sb, and 12 nota of the genotype, 109(2)68/2.4C; 11.1/NotchΔB2A3. Nota are from animals collected and heat-shocked at 18-20 hours after puparium formation (APF). Microchaete organs were counted from the region delineated by the left and right dorsocentral bristles. Averages for each set of data are displayed in bold print near the bottom of each column. To assess the confidence interval in comparing means from these two data sets, the standard error of the mean (SEM) is displayed in the final row. For average number of bristles from nota of flies lacking both the hs-*numb* and *NotchΔ* constructs as well as average number of bristles and average percent of double socket organs from *NotchΔ* flies, refer to a copy of this dissertation in the Jan Lab.

	109(2)68/2.4C; 11.1/TM3Sb							109(2)68/2.4C; 11.1/NotchΔ						
notum number	#total organs	#double sockets	#double hairs	#normal organs	%double socket	%double hairs		notum number	#total organs	#double sockets	#double hairs	#normal organs	%double socket	%double hairs
1	36	N/A	7	29	N/A	19.4		1	77	35	1	41	45.5	1.3
2	65	N/A	19	46	N/A	29.2		2	82	54	1	27	65.9	1.22
3	45	N/A	10	35	N/A	22.2		3	63	36	1	26	57.1	1.59
4	26	N/A	4	22	N/A	15.4		4	58	34	0	24	58.6	0
5	24	N/A	3	21	N/A	12.5		5	71	44	1	26	62	1.41
6	55	N/A	11	44	N/A	20		6	82	50	0	32	61	0
7	39	N/A	7	32	N/A	17.9		7	83	53	0	30	63.9	0
8	38	N/A	6	32	N/A	15.8		8	91	60	2	29	65.9	2.2
9	44	N/A	7	37	N/A	15.9		9	79	43	1	35	54.4	1.27
10	42	N/A	6	36	N/A	14.3		10	72	49	0	23	68.1	0
11	52	N/A	1	51	N/A	1.92		11	60	34	2	24	56.7	3.33
12	46	N/A	1	45	N/A	2.17		12	30	15	0	15	50	0
13	40	N/A	4	36	N/A	10								
14	46	N/A	10	36	N/A	21.7								
15	56	N/A	9	47	N/A	16.1								
16	51	N/A	4	47	N/A	7.84								
17	56	N/A	4	2	N/A	7.14								
18	36	N/A	2	34	N/A	5.56								
19	58	N/A	3	55	N/A	5.17								
20	53	N/A	2	51	N/A	3.77								
MEAN	**45.4**		**6**	**36.9**		**13.2**		**MEAN**	**70.7**	**42.3**	**0.75**	**27.7**	**59.1**	**1.03**
SEM	**2.31**		**0.94**	**2.71**		**1.65**		**SEM**	**4.7**	**3.53**	**0.22**	**1.89**	**1.96**	**0.31**

TABLE 3.1

CONCLUSION

SUMMARY AND PROSPECTUS

This thesis has focused on the role of the gene, *numb*, in specifying cell fate in the developing external sensory organ. The most significant aspect of this work is the demonstration that intrinsic differences between daughter cells can direct cell fate in a post embryonic division. That is, asymmetrically localized determinants are not exclusive to early embryonic divisions. Moreover, this mechanism of generating two unequal cells appears to be used in at least two and possibly all three divisions that give rise to es organ cells, suggesting that *numb* may impinge on a more general mechanism of establishing asymmetry within a cell. This expectation is enhanced by the implicated role of *numb* in asymmetric divisions within the chordotonal organ cell lineage as well as in the unequal divisions of the neuroblast.

Loss of *numb* function has previously been shown to cause the neuron and sheath cell to differentiate as outer support cells (Uemura et al., 1989). Examination of *numb* mutant clones in the adult reveals that these four cells all differentiate as sockets (Rhyu et al., 1994). Taken together with the phenotypes obtained when numb expression is induced at various points during es organ formation, the four-socket phenotype can be interpreted as a need for *numb* to specify IIb cell fate and hair cell fate. A role for *numb* in the neuron versus sheath fate is anticipated but has not been determined. This model can be simplified to show the need for *numb* in IIb-like versus IIa-like fates.

How is numb localized?

Overexpression of numb in both daughter cells yields fate transformations that are reciprocal to the loss of function phenotype. This suggests that numb recognizes and responds to a pre-existing asymmetry, rather than generating the

asymmetry itself. The origin of this asymmetry is a critical question that remains to be answered. The asymmetric localization of numb in the predivisional cell may occur in response to a localized signal from the environment. Alternatively, numb localization may reflect a purely intrinsic asymmetry that is extant in the predivisional cell.

Obvious sources for external cues are the mesoderm and the epidermis, which appose the neuroblast layer. Since localization of numb in neuroblasts is still observed in *twist*$^{-}$*snail*$^{-}$ embryos which lack mesoderm, it seems unlikely that an external signal to localize numb would emanate from this tissue. Although numb crescents are also visible in *crumbs* mutant embryos, which cannot maintain apical-basal polarity [J. Knoblich, unpublished data], it is still possible that neuroblasts receive a signal from the epidermis before polarity of the epithelium is lost.

In recent experiments by Juergen Knoblich, numb can be seen to localize to the region of the membrane that directly overlies one of the two centrosomes in a neuroblast cell. In *pebble* mutants, neuroblasts fail to complete cytokinesis after nuclear replication, resulting in cells that contain four centrosomes (Hime and Saint, 1992; Lehner, 1992). Juergen has found that, in *pebble* embryos, numb localizes to two distinct crescents that overlie the two adjacent centrosomes which are nearest to the mesoderm. Although a localized external signal cannot be ruled out, these experiments suggest that numb localization is a consequence of an intrinsic difference between the two centrosomes of the neuroblast. A difference in centrosomes might, for instance, manifest in unique cytoskeletal characteristics above one centrosome (Bornens, 1991). Regulation of the cytoskeleton is a function that has been proposed for PP2A (Healy et al., 1991; Ronne et al., 1991). It would be interesting to identify the component(s)

that constitute a difference between centrosomes and to understand the local effects of such a distinction.

If localization of numb is not directed by extrinsic signals, an important question is how a cell might intrinsically generate asymmetry. Because neuroblasts and SOP cells arise from epidermal cells which themselves have polarity, it is appealing to imagine that the asymmetry within the neuroblast is a remnant of the polarity of the epidermal cell. A model which invokes the epidermal history of the neuroblast must, however, account for the observation that, while neuroblasts divide perpendicular to the epidermal plane, SOP divisions are parallel to this plane. Thus, a model wherein numb localizes to the side of the neuroblast membrane that was previously basal would not explain how SOP cells localize numb to a portion of what was the lateral membrane of the epidermal cell.

Illuminating examples of how intrinsic polarity is established may be provided by studies in unicellular organisms such as yeast and bacteria. In these cases, it has been proposed that the site of the previous division leaves a scar which serves as a polar reference point for the new cell (Madden and Snyder, 1992; Maddock et al., 1993).

Another question with respect to numb localization is how it is coordinated with the plane of cell division so that the numb crescent segregates to one daughter rather than being split between the two cells. Assuming that numb responds to an intrinsic difference between the centrosomes, it can be concluded that the position of centrosome determines the plane of division, and localization of numb is secondary to the establishment of this axis.

Which cell inherits numb?

If *numb* is autonomous, it would direct IIb cell fate in the cell that inherits it; If *numb* is non-autonomous, it would go to IIa cells and signal the sister cell to become a IIb cell. Due to the lack of an independent marker that distinguishes the IIa cell from the IIb cell while numb is expressed, it has not been possible to determine which cell inherits the protein. An alternative strategy might be to generate mosaic clones to analyze which sister cell requires numb activity. Unfortunately, this experiment is not feasable. Since numb becomes localized in a crescent during prophase of mitosis, a mitotic recombination event during the division of the SOP cell would not influence the asymmetric segregation of numb protein. Absolute determination of *numb* autonomy may rely on the discovery of a marker which turns on early in one of the two daughter cells. Considering the limited time in which division and differentiation occur for es cells, I favor the simpler model where numb is inherited by the IIb.

Signaling downstream of numb

Once the numb protein segregates to one daughter, how does it influence the fate of that cell? The current data suggest that *N*, *Dl*, *neu*, *Su(H)* and *H*, in addition to *numb*, act to specify cell fates in each of the three divisions that give rise to es cells. These genes are known to interact in a common signaling pathway which transduces a signal from a neighboring cell. Thus, the function of inherited numb in these same divisions must be reconciled with the existence of the intercellular signaling pathway. Assuming that numb goes to the IIb cell, a key question regarding numb action, as discussed in Chapter 4, is whether numb activates a given fate program in this cell, or if numb functions to antagonize the inhibitory role of the Notch /Delta signaling system.

In support of numb's influence on the Notch signaling pathway, Shuwen Wang has discovered strong genetic interactions between *numb* and *Suppressor*

of Hairless, as well as *numb* and *Hairless*. The Delta-Notch-Su(H) cell culture assay used by Fortini et al. may be a valuable system in which to work out the biochemistry that underlies the genetic observations. Since numb protein is membrane associated, it might be expected to prevent nuclear localization of Su(H) by impeding Notch activation at the membrane. Shuwen is currently trying to identify other genes involved in signaling downstream of *numb* by isolating suppressors and enhancers of a viable reduction-of-function *numb* allele.

Is numb a determinant?

A determinant is defined as a protein which induces a specific developmental fate in the cell that inherits it. While the inheritance of numb enables one cell to be distinct from its sister cell, it does not dictate a single fate program. This is illustrated by the observation that the IIb cell and hair cell may both inherit numb, yet they differentiate distinctly. There must, therefore, be other factors that determine specific fates after numb exerts its function. It should be noted that, although numb is not a determinant, per se, determinative factors could still recognize asymmetry in a predivisional cell and become asymmetrically localized.

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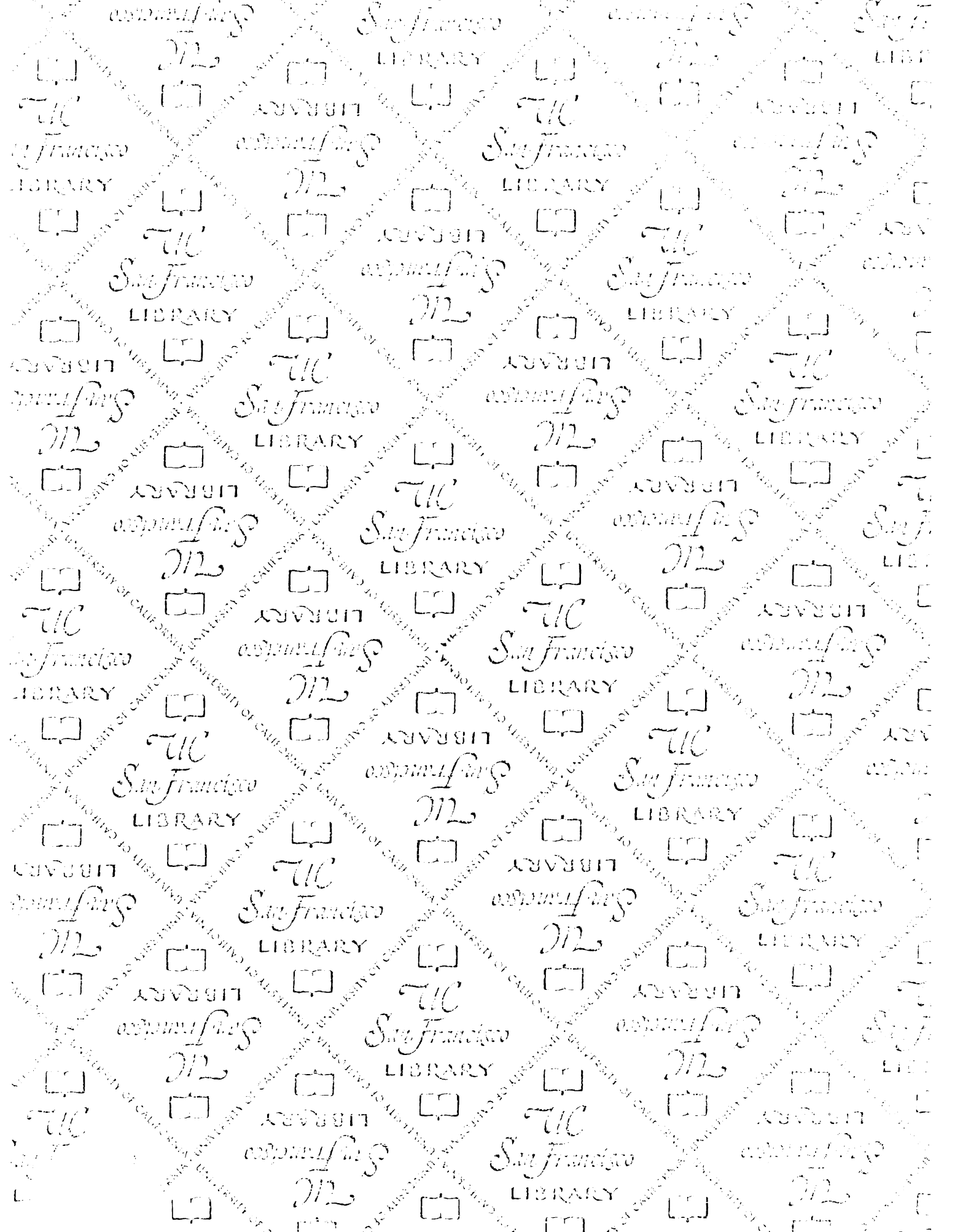
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