ROLES OF IMMUNOGLOBULIN DOMAIN PROTEINS ECHINOID AND FRIEND-OF-ECHINOID IN DROSOPHILA NEUROGENESIS

DISSERTATION

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By

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ABSTRACT

During neurogenesis in *Drosophila*, groups of ectodermal cells are endowed with the capacity to develop into neuronal precursors. The Notch signaling pathway is required to limit the neuronal potential to one or two cells within each group. Loss of genes of the Notch signaling pathway results in a neurogenic phenotype: hyperplasia of the nervous system accompanied by a parallel loss of epidermis.

Echinoid (Ed), a cell membrane associated Immunoglobulin C2-type protein has previously been shown to be a negative regulator of the EGFR pathway during eye development. Work from our lab has shown that Ed has a role in restricting neurogenic potential during embryonic neurogenesis. I have extended the functional and genetic analysis of Ed. Loss of *ed* suppresses the lack of neuronal elements caused by ectopic activation of the Notch signaling pathway. Using a temperature sensitive allele of *ed*, I show that Ed is required to suppress sensory bristles and for proper wing vein specification during adult development. In these processes also, *ed* acts in close concert with genes of the Notch signaling pathway. Overexpression of the membrane-tethered extracellular region of Ed results in a dominant-negative phenotype. This phenotype is suppressed by overexpression of Enhancer of split m7 {E(spl)} and enhanced by overexpression of Delta. Thus Ed interacts synergistically with the Notch signaling pathway.
I have identified a paralog of Ed, Friend-of-echinoid (Fred). *fred* function was examined in transgenic flies using inducible RNAi. Suppression of *fred* in the developing wing disc results in specification of ectopic SOPs, additional microchaeta and cell death. These phenotypes can be suppressed by increasing the activity of the Notch signaling pathway. Dosage-sensitive genetic interaction suggests a close functional relationship between *fred* and *ed*.

Microarray analysis of *fred* RNAi discs revealed a number of genes that are misregulated in the absence of Fred activity. Changes in the transcript levels predicted by the microarray data were validated by performing *in situ* hybridizations. One of the genes that is downregulated is *pannier* (*pnr*). Genetic interaction experiments suggest that Pnr cooperates with Fred to suppress neurogenesis in the apparently non-neurogenic regions of the wing disc.
Dedicated to my family
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\text{icd})$. $N
\text{icd}$ translocates to the nucleus where in a complex with Su(H) and Mam, it activates the expression of bHLH transcriptional repressors of the E(spl) complex. These bHLH E(spl) proteins directly suppress the expression of the proneural genes. Thus a cell in which the Notch signal is activated, the levels of genes of the AS-C decreases. Since the proneural proteins are required for the realization of neuronal potential, N signal receiving cells cannot become neural precursors and revert back to the default epidermal precursor cell fate

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4.1: Genomic organization and sequence of \textit{fred}. (A) \textit{fred} is located on chromosome arm 2L at 24D2 approx. 100kb upstream of \textit{ed}. Arrows indicate the direction of transcription. (B) \textit{fred} cDNA clones. Filled boxes represent the \textit{fred} open reading frame (ORF), while the open boxes indicate untranslated regions (UTR). The exon/intron arrangement is indicated. The introns are not drawn to scale. (a) \textit{fred} extendedCG3390 cDNA has an ORF of 3594bp, a 378bp 5'UTR and a 757bp long 3'UTR. Exons I-IX are 407bp, 202bp, 260bp, 198bp, 2232bp, 674bp, 367bp, 348bp and 63bp long. Introns 1-8 are 42,694bp, 16,450bp, 272bp, 9514bp, 107bp, 1684bp, 3392bp and 5401bp long. (b) \textit{fred} SD cDNA clone. 5'UTR is 5324bp long, the longest ORF is 2275bp long and is followed by a 2596bp long 3'UTR. (C) Predicted amino acid sequence of Fred. The seven IgC2 domains and the two Fn III domains are underlined. Transmembrane region (TM) is boxed. The C-terminal region is 188 amino acids long

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CHAPTER 1

Introduction

The development of an organism from a single cell to a multicellular three-dimensional structure has fascinated biologists. The building of an organism of characteristic shape and size is the result of coordinated gene action that directs developmental fate of the individual cells of the organism. Not surprisingly a lot of effort is being invested into deciphering signaling pathways that determine cell fate. Intrinsic, cell-autonomous factors as well as nonautonomous or extrinsic signals that can be short-range or long-range coordinate to specify cell fates. How intrinsic and extrinsic factors are integrated to specify cell fates is a central question of developmental biology.

Various model organisms have been used to study cell fate specification. The fruit fly, *Drosophila melanogaster* is my model organism of choice. Its advantageous features include a short generation time, ease of maintenance, sophisticated genetics, a sequenced genome, easy availability of fly stocks and *Drosophila* biologicals. Our lab focuses on the development of the embryonic central nervous system (CNS) as well as the adult peripheral nervous system (PNS) to explore mechanisms of cell fate specification.
CELLULAR ASPECTS OF NEUROGENESIS

Introduction to embryonic central nervous system

The CNS of insects arises from the proliferation of neural precursors called neuroblasts. Neuroblasts develop from a special neurogenic region, the neuroectoderm. Cells of the neuroectoderm have a choice to develop as neuroblasts or as epidermal precursors called epidermoblasts. Eventually, however, about 1500 out of 2000 neuroectodermal cells develop as epidermoblasts and only 500 become neuroblasts. Due to its apparent simplicity, the decision of neuroectodermal cell to adopt neural or epidermal fate is a good model system for exploring mechanisms of origin of cell diversity (Campos-Ortega, 1993).

Formation of neurogenic ectoderm

In wild-type Drosophila melanogaster, the neuroectoderm become morphologically distinct during the initial phase of germ-band elongation at stage 8 (3.4-4.2hrs after egg laying, AEL, at 25°C). Within the segmented germ band, the ectoderm differentiates a medial area with large cuboidal cells (Hartenstein, 1984). This medial sector is the ventral neuroectoderm (VNE) from which the ventral nerve cord and ventral epidermis will develop (Technau, 1985). Virtually all cells of the VNE enlarge to become conspicuously different from cells of the dorsal epidermal anlage. Once the neuroblasts have been determined and they segregate into the interior of the embryo, the remaining cells of the VNE shrink (Hartenstein, 1984).
Segregation of neuroblasts

Segregation of neuroblasts occurs over a time frame of three hours and is discontinuous, proceeding in five discrete waves, giving rise to five subpopulation of neuroblasts, called SI-SV neuroblasts (Campos-Ortega, 1985; Doe, 1992). Selected single neuroblasts within the VNE undergo conspicuous cell shape changes, leave the outer layer, and move internally to become located between the mesoderm and the ectoderm, where they form the neural primordium (Figure 1.1). The prospective epidermal progenitor cells also show characteristic cell shape changes. The neuroectodermal cells immediately adjacent to each of the SI neuroblasts establish physical contact with neuroblasts via long basal processes that transiently surround the segregated neuroblasts, forming a sort of sheath. Eventually, the ensheathing processes are retracted and the epidermoblasts diminish in size. Thus, sufficient contact exists between the neuroectodermal cells both before and during cell fate specification to enable cellular interactions to occur (Poulson, 1950).

Once the neuroblasts have been specified, each neuroblast divides asymmetrically, to give rise to a neuroblast and a ganglion mother cell (GMC), in a stem cell fashion. The neuroblast remains just internal to the epidermis and the GMC is pushed into the interior of the embryo. Each neuroblast divides five times on average. Each of the GMC divides to generate a pair of post-mitotic neurons. Thus the division of the neuroblasts generates the three-dimensional CNS from the two-dimensional neuroblast array (Goodman, 1993).
Introduction to the adult peripheral nervous system

In addition to the embryonic CNS formation, the generation of the external sensory organs, that comprise part of the adult peripheral nervous system, has been studied extensively to decipher cell-cell communication processes as well as intrinsic mechanisms of cell fate determination (Jan and Jan, 1993b).

*Drosophila* is holometabolous and has both a larval and an adult PNS. The larval PNS forms during embryogenesis and most of the larval sensory neurons degenerate during pupation. Adult PNS forms *de novo* during third instar larval stage and pupal development (Jan and Jan, 1993b).

External sensory organs

The dorsal thorax of *Drosophila* is covered with sensory bristles, which are part of the peripheral nervous system (PNS). Dorsal thorax is also referred to as notum. The large bristles, or macrochaeta, occupy a stereotyped position and occur in precise numbers. On each of the two halves of the notum, heminotum, occur eleven macrochaeta. The small bristles, or microchaeta, are arranged in rows with uniform density (Figure 1.2B). The arrangement of these sensory organs provides a classical model to study cell fate specification and patterning (Jan and Jan, 1993b). Each adult external sensory organ is composed of five cells: neuron, sheath cell, socket cell, hair cell and a glial cell (Bailey and Posakony, 1995; Jennings et al., 1994; Kim et al., 1996; Lecourtois and Schweisguth, 1995). Two of these components are exposed to the external environment: the mechanosensory structure called hair or shaft formed from the trichogen cell and a socket formed by the tormogen cell. The other two components of the external sensory organ are
internal: one neuron, with a single dendrite innervating the hair cell and an axon projecting into the CNS, and a support cell (thecogen cell or sheath cell) that surrounds part of the neuron (Jan and Jan, 1993b).

**Genesis of external sensory organ**

An external sensory organ arises from a sensory organ precursor (SOP) (Bodmer et al., 1989; Hartenstein and Posakony, 1989). During third instar larval and early pupal stages, SOPs arise in the imaginal wing disc, the larval epithelia that gives rise to the dorsal thorax epidermis and wings in the adult (Cubas et al., 1991; Huang et al., 1991). Thus, the position and number of bristles are determined by those of the SOPs (Figure 1.2A). The different SOPs appear in a strict sequence over a time span of more than 35 hours, during most of the third larval instar and the first ten hours after puparium formation (Huang et al., 1991). The SOP divides asymmetrically to produce two secondary precursors, IIa and IIb. The IIb cell again divides asymmetrically to generate a precursor IIIb and a glia cell that migrates away (Gho et al., 1999; Reddy and Rodrigues, 1999). The IIa cell divides asymmetrically to generate a hair cell and a socket cell (the two outer support cells). Finally, the IIIb precursor divides to generate a neuron and a sheath cell (Hartenstein and Posakony, 1990). The delay between the formation of a SOP and its first mitotic division ranges from 30 hours, in the case of the earliest SOP, to 2 hours for the last SOP (Huang, 1990).

The early events of neural precursor development both in embryogenesis and adult PNS are mediated by similar sets of genes. At both stages of neural development the separation of neural and epidermal progenitor cells is controlled by two groups of
genes, the proneural genes that endow cells with neural potential and neurogenic genes, that inhibit cells from adopting a neural precursor cell fate. Prepattern genes are responsible for setting up the coordinates at which the proneural genes are expressed.

GENETIC ASPECTS OF NEUROGENESIS

Proneural genes: Endowing cells with potential to become neural precursors

The term proneural was coined to describe genes that define a state that makes cells competent to become neural precursors (Ghysen and O'Kane, 1989; Romani et al., 1989; Simpson and Carteret, 1990). Genes of the *achaete-scute* complex (AS-C) and *daughterless* (*da*) belong to this group. The AS-C consists of four bHLH genes *achaete* (*ac*), *scute* (*sc*), *lethal of scute* (*l'sc*) and *asense* (*ase*), which are separated by long regions of nontranscribed DNA (Campuzano and Modolell, 1992). Proteins encoded by *ac*, *sc* and *l'sc* are first detected in a cluster of four to six ectodermal cells in stereotyped positions in the VNE (Cabrera et al., 1987; Martin-Bermudo et al., 1991). These groups of cells in the neurogenic region expressing the proneural genes are called proneural clusters (Ghysen and Dambly-Chaudiere, 1989). One neuroblast segregates from each cluster and retains proneural gene expression and rest of the cells of the cluster lose the expression and go on to an epidermal cell fate (Cabrera, 1990; Cabrera et al., 1987; Martin-Bermudo et al., 1991; Skeath and Carroll, 1992). *l'sc* is mainly dedicated to the formation of proneural clusters in the embryonic VNE (Martin-Bermudo et al., 1991). *ase* is expressed in all singled out neural precursors and is required to ensure neural cell fate (Bertrand et al., 2002; Campuzano and Modolell, 1992).
Proneural genes *ac* and *sc* are also required for external sensory organ formation in the notum of the adult fly (Ghysen and Dambly-Chaudiere, 1989; Jan and Jan, 1993b). The SOPs that generate the macrochaetae and microchaetae are specified from the epidermal tissue by the action *achaete* (*ac*) and *scute* (*sc*) (Campuzano and Modolell, 1992; Jan and Jan, 1993b). These two proteins function *in vivo* as heterodimers with the bHLH protein Daughterless (Murre et al., 1989; Vaessin et al., 1990; Van Doren et al., 1991). These proneural genes define the proneural cluster, a small group (10-20 cells) of ectodermal cells in the wing disc, which have the potential to develop into SOPs (Figure 1.2A) (Campuzano and Modolell, 1992; Modolell, 1997). The expression of *ac* and *sc* in proneural clusters of the imaginal disc is regulated by a combinatorial prepatterning of transcription factors that interact with the enhancer regions of these genes (Ghysen and Dambly-Chaudiere, 1988; Gomez-Skarmeta et al., 2003). The proneural genes presumably commit cells to become SOPs by activating downstream genes that participate in the neural differentiation program.

**Prepattern genes: Setting up the coordinates**

**Prepatterning during embryonic neurogenesis**

At the end of cellular blastoderm, the embryo is already subdivided into parasegments along the anterior-posterior (A-P) axis via the action of segmentation genes (Reviewed in (Bhat, 1999). Another set of genes specifies the dorsoventral (D-V) axis and divides the trunk region of the embryo into a series of longitudinal stripes (Chasan, 1993). Certain tissue type develops from each stripe. Thus, the ventral domain develops into the mesoderm, the ventrolateral domain gives rise to the embryonic brain, embryonic
CNS, PNS and ventral epidermis, and the dorsolateral domain develops into PNS and dorsal epidermis (Mayer and Nusslein-Volhard, 1988). The boundaries of proneural gene expression appear to be set up by the expression of pair rule genes such as \textit{fushi-tarazu} and \textit{even-skipped} and segment polarity genes along the A-P axis (Doe et al., 1988a; Doe et al., 1988b; Patel et al., 1989), and by zygotic D-V genes, such as \textit{dorsal} and \textit{decapentaplegic (dpp)}, along the D-V axis (Skeath and Carroll, 1992; Skeath et al., 1992). The transcription factor Dorsal is expressed in a gradient along the D-V axis with the highest concentration in the ventral-most cells. Dorsal determines the position and the extent of the VNE by activating the expression of the neural prepatterning genes and by antagonizing the activity of Dpp, a dorsal epidermal fate inducer (Rusch and Levine, 1996). The opposing activities of Dorsal and Dpp control the expression of the prepattern genes: \textit{ventral nervous system defective (vnd)}, \textit{intermediate neuroblasts defective (ind)}, \textit{muscle segment homeobox (msh)}, \textit{SoxNeuro (SoxN)} and \textit{Dichaete (D)} (Cowden and Levine, 2003; Oh et al., 2002). \textit{vnd, ind} and \textit{msh} genes establish the basic D-V subdivisions of the VNE (Reviewed in (Cornell and Ohlen, 2000; Skeath, 1999)). These genes are expressed in adjacent, non-overlapping longitudinal ventral, intermediate and dorsal domains in the VNE from which neuroblasts delaminate in five successive waves (Chu et al., 1998; Isshiki et al., 1997; McDonald et al., 1998; Weiss et al., 1998; Yagi et al., 1998). In the intermediate column after SII neuroblast segregation, Ind becomes dispensable for neuroblast specification and SoxN and Dichaete are required (Buescher et al., 2002; Cremazy et al., 2000; Overton et al., 2002; Zhao and Skeath, 2002). Vnd activates \textit{ac} expression in the ventral column; SoxN activates \textit{ac} in the dorsal column and reinforces it in the ventral column (Buescher et al., 2002; Overton et al., 2002; Skeath et
al., 1994). In the intermediate column Ind represses ac and activates l’sc (Weiss et al., 1998). Dichaete represses ac in the intermediate column but has no effect on the ac expression in the ventral column (Overton et al., 2002; Zhao and Skeath, 2002). Thus the expression pattern of the proneural genes is prefigured by the action of several prepatterning genes (Reviewed in (Gomez-Skarmeta et al., 2003)).

**Prepattern formation in wing discs**

Similar to the embryonic VNE, a combination of prepatterning genes determines the positions of proneural clusters and hence the positions of SOPs in the larval wing disc (Jan and Jan, 1993b). Pannier (Pnr), a GATA transcription factor expressed in the medial region of the wing disc is required for the specification of the Dorsocentral (DC) and Scutellar (SC) proneural clusters (Figure 1.4A) (Heitzler et al., 1996a; Ramain et al., 1993). Reduced function of Pnr leads to loss of expression of ac-sc in the DC and SC proneural clusters, and leads to the absence of the corresponding SOPs (Haenlin et al., 1997). U-shaped (Ush), an antagonist of Pnr, is expressed in pattern overlapping that of Pnr. Ush physically interacts with Pnr, and can modulate its activity to change it from an activator to a repressor (Cubadda et al., 1997). Recently, an enhancer that drives ac-sc expression in the DC proneural cluster has been isolated. There is strong evidence that Pnr binds to this DC enhancer directly and that it can activate transcription. So, Pnr appears to be a direct activator of ac-sc in the DC cluster. This cluster is located between the borders of Pnr and Ush expression, here only Pnr is present and Ush is absent. Thus the availability of the activator Pnr establishes its lateral limit, whereas the presence of the Pnr/Ush repressor complex seems to set its medial limit. These conclusions are
supported by the observation that the DC cluster expands into the Ush domain when the wild type Pnr is replaced by a mutant Pnr that cannot bind Ush to form the repressor complex (García-García et al., 1999). Role of Pnr in the formation of the SC proneural cluster has not been so well studied. However, based on the loss of ac-sc expression from the medial region of the wing disc upon reduced Pnr function, Pnr is considered a part of the prepatteren that leads to the establishment of proneural clusters at stereotyped positions (Reviewed in (Gomez-Skarmeta et al., 2003)).

The Iroquois complex genes have been described as constituents of the prepatteren that directs ac-sc expression in the lateral region of the presumptive notum (Reviewed in (Cavodeassi et al., 2001)). Iroquois (Iro) genes were discovered in Drosophila in the course of a mutagenesis screen designed to identify genes that affect the patterning of external sensory organs (Dambly-Chaudiere and Leyns, 1992). The first mutant allele recovered (iro^1) suppressed all of the lateral bristles of the dorsal mesothorax (notum), leaving only a wide band of sensory bristles in the medial region of the notum. This pattern resembled the hairstyle of the Iroquois American Indians- hence the name of the locus (Leyns et al., 1996). It was further shown that the suppression of bristles was a result of failure of proneural genes of the AS-C to be expressed in the lateral region of the wing disc epithelium. As a consequence the SOPs were not formed (Leyns et al., 1996). These results suggested that the Iro locus might encode a factor(s) that allowed the expression of the AS-C in the presumptive lateral notum. Molecular characterization of the locus identified three genes that comprise the Iroquois complex (Iro-C). The individual genes, named araucan (ara), caupolican (caup) and mirror (mirr) (Gomez-Skarmeta and Modolell, 1996; McNeill et al., 1997), span approximately 130kb of DNA
(Netter et al., 1998). Ara and Caup are closely related whereas Mirr is more divergent (Cavodeassi et al., 2001). All three proteins have homeodomain and Iro box (possibly involved in protein-protein interactions) and constitute the family of homeodomain proteins within the TALE class (Burglin, 1997). Ara and Caup have identical patterns of expression apparently due to their sharing of common enhancers within the Iro-C (Gomez-Skarmeta and Modolell, 1996). Mirr shows a very similar pattern of expression and may share parts of these enhancers (McNeill et al., 1997). In a third instar larval wing disc, all three genes are expressed in lateral region of the notum (Figure 1.4B). In the presumptive notum of the *iro*¹ flies (molecular lesion in *iro*¹ results from a breakpoint in the *caup* locus), the expression of *ara* and *caup* is strongly decreased (Gomez-Skarmeta and Modolell, 1996). This most probably leads to a lack of *ac-sc* expression in the lateral notum and, as a result, the absence of sensory organs (Leyns et al., 1996). The depletion of Mirr alone also removes two out of the seven lateral macrochaeta and it genetically interacts with *iro*¹ and other *Iro* recessive alleles (Kehl et al., 1998). Thus, the three Iro-C proteins appear to promote the expression of *ac-sc* and given the functional redundancy between them, a certain level of overall Iro-C function is probably required for *ac-sc* expression (Cavodeassi et al., 2001). As the Ara protein binds in vitro to an evolutionarily conserved, functionally indispensable sequence of an *ac-sc* regulatory element, it has been proposed that the Iro-C proteins directly activate these proneural genes (Gomez-Skarmeta and Modolell, 1996). However, overexpression of a chimeric protein containing the Ara homeodomain fused to the Engrailed repressor domain, which should function as a repressor, expanded the proneural cluster governed by that regulatory element. This expansion was similar to that obtained by overexpressing wild-
type Ara, suggesting that this, and other Iro proteins, act as transcriptional repressors. Hence, their upregulation of ac-sc expression should be indirect (Cavodeassi et al., 2001).

Once the pattern of proneural genes expression is established through a combinatorial action of prepatterning genes, the function of the neurogenic genes is required to downregulate the expression of the proneural genes. Thus within every proneural cluster of cells, most of the cells lose proneural protein expression except for one or two future neural precursor cells. This selection of a neural precursor cell from a group of equipotent proneural cells occurs via the process of lateral inhibition (Cabrera, 1990; Simpson, 1990). Lateral inhibition is mediated by the members of the Notch signaling pathway (Artavanis-Tsakonas et al., 1995; Artavanis-Tsakonas et al., 1999). Genes of the Notch pathway are classified as neurogenic as reduced function of any of these genes results in a hyperplasia of the nervous system that is accompanied by loss of the ventral epidermis (Lehmann et al., 1983). Based on their phenotype Notch pathway genes are described as neurogenic genes; based on their function these can be classified as anti-neural genes as they inhibit neurogenesis. Lateral inhibition is used both during the selection of neuroblasts in embryos as well as SOPs in larval wing disc.

**Neurogenic genes: Limiting neurogenesis**

Neurogenic genes have been shown to interact genetically in their function of inhibiting neural cell fate determination. Removing the function of any of the known zygotic neurogenic genes, Notch (N), Delta (Dl), Suppressor of Hairless (Su(H)), the Enhancer of split complex (E(spl)-C), mastermind (mam), neuralised (neu), big brain (bib), grouch (gro), shaggy (sgg) and presenilin (psn) results in a hyperplasia of the
embryonic CNS as well as the adult PNS (Artavanis-Tsakonas et al., 1999; Campos-Ortega, 1993). Although loss of any of these neurogenic genes causes neural hyperplasia, the severity of the phenotype differ. For example, bib null mutants only cause a two-fold increase in the number of sensory organs as opposed to the dramatic increase seen in Notch and Delta mutants. The basis of this difference in the phenotype was determined by an examination of the proneural clusters. In bib null mutants, only a subset of cells of the proneural cluster become SOPs, whereas in Notch or Delta mutants, almost all the cells of the proneural cluster retain their neurogenic potential and develop as SOPs. The bib mutant phenotype suggests that within the proneural cluster (PNC), only a few cells require bib function to suppress neurogenic potential, whereas each of the cells of the PNC require N and Dl for inhibition of neural cell fate (Rao et al., 1992). Moreover, Bib is required for selection of SOPs but not for specifying cell fates of the progenies of the SOP. In contrast N and Dl are required not only for SOP specification but also for the SOP progenies to assume correct cell fate (de Celis et al., 1991; Hartenstein and Posakony, 1990).

Neurogenic gene function is not limited to neural cell fate suppression. Development of multiple tissues, including eye, segmented appendages such as leg, mesoderm, muscle, somatogastric nervous system, wing and oogenesis requires neurogenic gene function (Artavanis-Tsakonas et al., 1995).

**Lateral inhibition: Notch signaling pathway**

A lateral inhibition model has been proposed to explain the process of selection of a single neural precursor from the cluster of equivalent proneural cells (Figure 1.6)
(Heitzler and Simpson, 1991; Jan and Jan, 1993a; Simpson, 1990; Simpson, 1997).

According to the current thinking, through a stochastic event, a single cell in the PNC, happens to have a slightly higher levels of the proneural genes. This cell can send an inhibitory signal to the surrounding cells and prevent them from adopting a neural precursor cell fate. Hence, the term-lateral inhibition. Proneural genes positively regulate the levels of the Notch (N) ligand, Delta (Dl) (Haenlin et al., 1994). The cell in the proneural cluster with a higher amount of the proneural proteins can send a stronger inhibitory signal via the increased expression of the transmembrane ligand Dl to the surrounding cells that receive the signal via the transmembrane receptor N. Upon activation, N undergoes Presenilin dependent processing which results in the release of the N intracellular domain (N \text{icd}) from the membrane (De Strooper et al., 1999; Struhl and Adachi, 1998; Struhl and Greenwald, 1999). N \text{icd} translocates to the nucleus where it forms a complex with the sequence-specific DNA binding protein Suppressor of Hairless \{Su(H)\} (Fortini and Artavanis-Tsakonas, 1994; Furukawa et al., 1992; Schweisguth and Posakony, 1992; Tamura et al., 1995) and Mastermind (Petcherski and Kimble, 2000), a transcriptional co-activator, to switch on the expression of multiple genes of the Enhancer of split \{E(spl)\} complex (Bailey and Posakony, 1995; Furukawa et al., 1995; Lecourtois and Schweisguth, 1995). This gene complex \{E(spl)m8, E(spl)m7, E(spl)m5, E(spl)m3, E(spl)m\delta, E(spl)m\gamma and E(spl)m\beta\} encodes seven closely related proteins of the bHLH family of transcription factors (Delidakis and Artavanis-Tsakonas, 1992; Klambt et al., 1989; Knust et al., 1992). E (spl) bHLH proteins act as transcriptional repressors in a complex with the co-repressor protein Groucho (Delidakis and Artavanis-Tsakonas, 1992; Fisher and Caudy, 1998) and down-regulate ac and sc expression.
leading to suppression of neural precursor cell fate in the cell receiving the signal (Heitzler et al., 1996a) (Figure 1.6). In the absence of N signaling many cells of the proneural cluster develop as neural precursor cells (Dietrich and Campos-Ortega, 1984; Schweisguth et al., 1996). In contrast, the absence of the proneural genes results in an almost complete lack of neural precursors (Figure 1.6)(Cabrera et al., 1987; Jimenez and Campos-Ortega, 1990).

**EGFR signaling is required for the formation of external sensory organs**

Epidermal Growth Factor Receptor (EGFR) signaling has also been implicated in the process of SOP specification (Clifford and Schupbach, 1989). EGFR signaling is transduced by the Ras/Raf/MAPK signaling cascade. Hypomorphic mutations and expression of dominant negative constructs of the EGFR gene result in the loss of SOPs (Culi et al., 2001; Diaz-Benjumea and Garcia-Bellido, 1990); over-activity of the EGFR pathway results in differentiation of extra SOPs (Culi et al., 2001). The mechanism by which the EGFR pathway regulates SOP formation is not yet clear. It has been proposed that the EGFR pathway is necessary to promote increased accumulation of the Ac and Sc in the future SOP (Culi et al., 2001). When the EGFR signaling is reduced, the level of sc expression decreases, leading to absence of SOPs. Thus the Notch signaling pathway would work antagonistically to the EGFR pathway by repressing ac and sc via the E (spl) genes. Antagonism between the two pathways also regulates the differentiation of photoreceptors in the eye, where the Notch signaling pathway inhibits photoreceptor differentiation while the EGFR pathway promotes it (Freeman, 1996; Sun and Artavanis-Tsakonas, 1996; Tio and Moses, 1997; Xu and Rubin, 1993). Antagonism between the
two pathways also regulates recruitment of chordotonal sense organ precursors (zur Lage and Jarman, 1999).

**Echinoid and Friend-of-echinoid and their role in neurogenesis**

*echinoid* mutation, ed<sup>2B8</sup>, was recovered through an enhancer trap screen (Bier et al., 1989). *ed* was subsequently analyzed and cloned by A. Ahmed (Ahmed, 1999). *ed* mutant alleles were also independently identified as enhancers of Ellipse allele of EGFR and cloned (Bai et al., 2001). Echinoid is predicted to have seven Immunoglobulin C2 domains, two fibronectin type III domains, a transmembrane region followed by a 315 amino acid long intracellular region with no identifiable functional or structural motifs (Ahmed, 1999; Bai et al., 2001).

Immunoglobulin (Ig) C2 proteins possess a common domain structure (approximately 100 amino acids long), typically with two cysteines in each domain (separated by approximately 50 amino acids) as well as a highly conserved tryptophan residue. Additional conserved residues are located around the cysteine residues. The Immunoglobulin domain forms a three-dimensional structure called the immunoglobulin fold. The Immunoglobulin fold is a globular structure containing two β sheets, each consisting of three to four antiparallel β strands of five to ten amino acids each. Intrachain disulfide bonding between the conserved cysteine residues stabilizes the structure (Hortsch and Goodman, 1991). Ig C2 domain proteins commonly contain two to seven IgC2 domains and a variable number of Fibronectin (Fn) type III domain in their extracellular region (e.g. L1, neuroglian, Fasciclin II, Frazzled). Fn III domain is approximately 90 amino acids long. It was first identified as one of the major repeat
domain in mammalian fibronectin (Hynes, 1986). Although Fn III domains typically exhibit less extensive homology between one another than do Ig domains, they have several amino acids that are well conserved, these include a tryptophan and a tyrosine residue that are separated by 45 to 55 amino acids (Hortsch and Goodman, 1991). The Ig domain proteins are frequently involved in homophilic as well as heterophilic protein-protein interactions (Bixby and Harris, 1991). Many of these proteins are also involved in signal transduction. Ig C2 domain proteins are either integral membrane proteins or linked to the membrane through a glycoprophosphatidyl inositol (GPI) anchor (Bixby and Harris, 1991). Secreted Ig C2 domain proteins like Amalgam and Vein have also been reported (Schnepp et al., 1996; Seeger et al., 1988).

In the following chapters, I will describe the functional characterization of Echinoid and show that it is required to suppress neurogenesis in embryos and adults. I have extended A. Ahmed’s observations of genetic interactions between ed and genes of the N signaling pathway. In course of cloning ed, a parologue of ed, friend-of-echinoid (fred) was discovered (Ahmed, 1999). I have analyzed fred’s function by knocking down fred RNA via inducible RNAi. Similar to ed, fred is required to prevent neural precursor cell fate specification. ed and fred show genetic interactions with genes of the Notch signaling pathway and with each other, suggesting a functional synergy. The fred knockdown phenotype is distinct from the ed neurogenic phenotype and from that of the genes of the Notch signaling pathway. The extra SOPs arise in regions of the wing disc where no proneural cluster and hence no neural potential exists. To explore the mechanistic basis of this phenotype, I have performed microarray analysis. Results from this analysis will be presented.
Figure 1. Early neurogenesis in *Drosophila melanogaster* embryo. Embryos at developmental stages 5, 8 and 9 (stages according to (Campos-Ortega, 1985)) are diagrammed. Lateral view is on left and a cross section is shown on the right. A stage 5 embryo shows specification of ventral neuroectoderm (VNE), procephalic neuroectoderm (PNE) and mesectoderm (mec). mec, a single row of cells on either side of the embryo, separates the mesoderm (ms) from the ventral neuroectoderm (VNE). By stage 8, mesoderm invaginates, positioning the VNE at the ventral region of the embryo. At this stage the cells of the VNE enlarge and neuroblasts delaminate from the VNE. In a stage 9 embryo, three rows of neuroblasts generated in three waves of determination and delamination are visible.
From (Hartenstein, 1993)

Figure 1. 1: Early neurogenesis in *Drosophila melanogaster* embryo
Figure 1. 2: Genesis of adult external sensory organs. Schematic of a wing imaginal disc is shown in (A). Medial and lateral parts of the wing disc that give rise to the medial and lateral part of the dorsal thorax or notum are indicated. Proneural clusters from which the dorsal thorax macrochaeta arise are shown. Circles within the proneural clusters indicate the SOPs. (B) Heminotum of the adult fly with the stereotyped arrangement of macrochaeta and microchaeta is shown. a-anterior; p-posterior; NP-notopleural; PS-presutural; SA-spuraalar; PA-postalar; DC-dorsocentral; SC-scutellar. Figures from (Calleja et al., 2002).
Figure 1.2: Genesis of adult external sensory organs. Figure adapted from (Calleja et al., 2002).
Figure 1. 3: Development of mechanosensory bristles. The SOP divides to generate two secondary precursor cells IIa and IIb. IIa divides to give rise to the socket and the hair cell. IIb divides to generate a third-order precursor cell and a glial cell. The glial cell migrates away and does not contribute to the formation of the sense organ (Gho et al., 1999). The IIIb cell divides further to give rise to the neuron and the sheath cell that protects the neuron. The IIb cell prevents IIa cell from acquiring the IIb fate by activating the N signaling pathway in IIa cell. Later the hair cell and the neuron prevent the socket cell and the sheath, respectively, from adopting the same fate by activating N signaling in the socket and sheath cell. Figure adapted from (Koelzer and Klein, 2003).
Figure 1. 3: Development of mechanosensory bristles. Figure adapted from (Koelzer and Klein, 2003).
Figure 1.4: Pannier and Iroquois complex proteins expression pattern (A) Part of the wing disc is shown. Pannier expression (in red) is limited to the medial part of the future dorsal thorax. Figure (A) is from (Calleja et al., 2000). The DC and SC proneural clusters arise in this region of the wing disc. The lateral part of the notum expresses genes of the Iroquois complex (green), visualized here with an antibody that recognizes both Ara and Caup. (B) A comparison of Iro-C proteins expression pattern to that of the proneural clusters. All the macrochaeta that arise in the lateral notum, emerge from proneural clusters in the Iro-C domain of expression. Figure (B) is from (Gomez-Skarmeta et al., 2003).
Figure 1. 4: Expression patterns of Pannier and Iroquois complex proteins. Figure (A) is from (Calleja et al., 2002) and (B) is from (Gomez-Skarmeta et al., 2003).
Figure 1. 5: Cell-cell communication during neurogenesis. The VNE is boxed in the schematic of a *Drosophila* embryo. In the VNE, groups of cells acquire the potential to develop into neuronal precursors via the expression proneural genes of the AS-C. Within a proneural cluster, only one or two cell retain the neuronal potential and remaining cell of the proneural cluster go on to develop as epidermal precursors. The neurogenic genes function to limit the neuronal potential by the process of lateral inhibition. In the absence of proneural gene function, almost no neuronal precursors are specified. In contrast, loss of neurogenic genes leads to a hyperplasia of the nervous system that is accompanied by loss of epidermal tissue.
Cell-to-Cell Communication During Neurogenesis

Prepattern genes

Proneural genes
Achaete-Scute Complex

Neurogenic genes
Notch, Delta, E(spl), mam, neu, bib

Phenotype of loss-of-function mutations

- neuronal precursors
- epidermal precursors

No (or too few) neuronal precursors
Too many neuronal precursors

Adapted from (Jan and Jan, 1993b)

Figure 1.5: Cell-cell communication during neurogenesis.
Figure 1.6: Components of the Notch signaling pathway. The future neural precursor cell (green) inhibits the surrounding cells (red) of the proneural cluster from adopting a neuronal precursor fate. The future neural precursor cell happens to have a higher level of the Ac-Sc. Ac-Sc activate the expression of Delta (Dl), the transmembrane ligand of Notch. Thus, this cell can hence send a stronger inhibitory signal to the surrounding cells. The surrounding cells receive the signal via the transmembrane receptor Notch (N). Upon binding to Dl, N undergoes a Psn dependent cleavage that releases the intracellular domain of N (N^{icd}). N^{icd} translocates to the nucleus where in a complex with Su(H) and Mam, it activates the expression of bHLH transcriptional repressors of the E(spl) complex. These bHLH E(spl) proteins directly suppress the expression of the proneural genes. Thus a cell in which the Notch signal is activated, the levels of genes of the AS-C decreases. Since the proneural proteins are required for the realization of neuronal potential, N signal receiving cells cannot become neural precursors and revert back to the default epidermal precursor cell fate.
Elements of the Notch Signaling Pathway

Adapted from Artavanis-Tsakonas, 1999.

Figure 1.6: Components of the Notch signaling pathway
CHAPTER 2

Materials and methods

Fixation of *Drosophila* embryo for antibody staining

Embryos were collected in a mesh basket and dechorionated for 5 minutes in 50% bleach. Fixation was carried out for 20 minutes in a mixture of 4% formaldehyde in 1X PBS and an equal volume of heptane. The aqueous fixative was replaced with methanol and the embryos were devitellinized by vigorous shaking. Unfixed embryos (the ones at the interphase of heptane and methanol), methanol and heptane were removed and the embryos were rinsed twice with methanol and three times with absolute ethanol before storing at 4°C in ethanol.

Antibody staining of whole mount embryos with HRP /fluorescent- conjugated secondary antibody

Fixed embryos were rehydrated gradually by replacing ethanol with PBT (1X PBT, 0.1% Triton X-100). After rehydration, the embryos were blocked by incubating in 1% BSA (in PBT) for an hour at room temperature. Proper dilution of primary antibody was added, and the embryos were incubated for 2 hours at room temperature or overnight at 4°C. After four washes with PBT, 15 minutes each time, the HRP-conjugated secondary antibody was added for 2 hours at room temperature. After four 15 minutes washes with PBT, the embryos were transferred to a 24-well plate in 400 μl of 1X PBT.
100 ul of DAB (Diaminobenzidine; 3mg/ml stock) was added to each well and incubated for 5 minutes. 500 ul PBT containing 0.03 % hydrogen peroxide (diluted fresh from 3% stock kept at 4°C) was added to each well. After the color developed to an appropriate intensity, the reaction was stopped by adding an equal volume of absolute ethanol. The embryos were dehydrated gradually into ethanol and the ethanol was replaced with xylene. The embryos were mounted in Permount. When a fluorescent secondary antibody was used, the incubation time was decreased to 1.5 hours and the washing was increased to 4 times 30 minutes each time. The embryos were protected from fluorescent light at all times. The embryos were mounted in Vectashield and visualized by confocal microscopy using a Bio-Rad MRC 1024 system.

**Preparation of Digoxigenin labeled RNA probes**

cDNA contracts were used as templates for generating digoxigenin labeled antisense RNA probes. 1 µg of cDNA construct was linearized using a proper restriction enzyme that would cut at a position opposite from the RNA polymerase transcription initiation site to be used for generating anti-sense RNA transcripts. Digoxigenin labeled RNA probe was generated using Digoxigenin RNA labeling kit from Roche. The transcription and labeling reactions were performed according to manufacturer’s specification. Linearized DNA was extracted with phenol, phenol/chloroform, and chloroform. DNA was precipitated with ethanol and washed twice with 75% ethanol. Air dried DNA was resuspended in 5 µl DEPC-treated water double-distilled water (ddH₂O). Mixture of 1 µl of 10X transcription buffer, 1 µl of 50 mM DTT, 1 µl of RNase inhibitor (20-40 U/µl), 1 µl of 10X dig-UTP/NTP RNA labeling mix and 1 µl of appropriate (T3,
T7 or SP RNA polymerase (10-20 U/µl) was added to the resuspended DNA. The reaction was incubated at 37°C for 2 hours. The probe was hydrolyzed in 15 µl of DEPC-treated ddH₂O and 25 µl of 2X carbonate buffer (120 mM Na₂CO₃ and 80 mM NaHCO₃) for 10 minutes. Hydrolysis reaction was stopped at appropriate time by adding 50 µl of stop solution (5 µl of 3M NaOAc and 45 µl of DEPC-treated ddH₂O). Probe was precipitated by adding 10 µl of 4M LiCl, 10 µl yeast tRNA (20mg/ml), and 2-3 volumes of ethanol and stored at -80°C for 20 minutes. The labeled RNA probe was spun down at 4°C, for 30 minutes at maximum speed and washed once with 75% ethanol. The air-dried RNA pellet was resuspended in 100 µl of hybridization buffer (50% formamide, 5X SSC, 0.1 mg/ml yeast tRNA, 0.05 mg/ml Heparin, 0.01% Tween-20 and ddH₂O) and stored at -20°C.

Fixation of Drosophila embryo for in situ hybridization

Embryos were collected and dechorionated in 50% bleach for 5 minutes. Embryos were fixed for 25 minutes in a mixture of 10% formaldehyde in 1X PBS, and equal volume of heptane. After replacing the aqueous fixative with methanol, embryos were devitellinized by vigorous shaking. Fixed embryos were rinsed twice with methanol and dehydrated with absolute ethanol and stored at -20°C at least one day before use.

RNA in situ hybridization of whole mount Drosophila embryos

100 µl of embryos (stored in ethanol at-20°C) fixed in 10% formaldehyde were transferred into an eppendorf tube and rinsed twice with ethanol. Embryos were treated with 50/50 (v/v) xylene/ethanol for 30 minutes at room temperature followed by five washes with ethanol. After two rinses with methanol, embryos were post-fixed in 50/50
methanol/5% formaldehyde in 1X PBT (1X PBT. 0.1% Tween-20) for 5 minutes, followed by incubating with 5% formaldehyde in 1X PBT at room temperature for 25 minutes. Embryos were rinsed five times with 1X PBT, and treated with 2 µl of Proteinase K (10 mg/ml) diluted in 500 µl of 1X PBT at room temperature for 5 minutes. The embryos were washed with 1X PBT twice quickly, and four times for 2 minutes each time to stop Proteinase K treatment. Embryos were post-fixed again with 5% formaldehyde in 1X PBT at room temperature for 25 minutes. After rinsing 5 times with 1X PBT, embryos were incubated in 50/50 1X PBT/hybridization solution at room temperature for 10 minutes, rinsed once in hybridization solution for 2 minutes, and pre-hybridized in hybridization solution for at least 2 hours at 55°C. Proper amount of RNA probe (usually 2 µl of probe in 40 µl hybridization solution per reaction for most probes) was heated at 85°C for 5 minutes and then chilled on ice. Chilled probe was added onto the embryos. Hybridization reaction was allowed to proceed for 12-18 hours at 55°C. After removing the probe, embryos were rinsed once in hybridization solution and incubated in hybridization solution for 1 hour at 55°C, followed by four washes with hybridization solution at 55°C for 30 minutes each. Embryos were incubated 3 times in hybridization solution/1X PBT with decreasing ratio of hybridization solution at 55°C for 20 minutes each, followed by four washes with 1X PBT for 20 minutes at room temperature. Anti-Dig antibody (1:4000 final dilution) was pre-absorbed as described at 1:200 dilution. For preabsorbing antibody, embryos fixed for antibody staining (4% formaldehyde) were rehydrated with gradual washes into PBT. The embryos were blocked in 1% BSA (in PBT) for an hour. Anti-Dig antibody diluted at 1:200 in 1% BSA was added to the embryos and incubated for an hour. The supernatant containing the
preabsorbed antibody was transferred to a new tube and stored at 4°C. The preabsorbed antibody was used within 24 hrs. For each reaction, 25 µl of pre-absorbed anti-Dig antibody was diluted in 500 µl of 1X PBT and added on to the embryos to incubate overnight at 4°C. Embryos were washed four times with 1X PBT at room temperature for 20 minutes each and two times with alkaline phosphatase (AP) staining buffer (100mM Tris pH 9.5, 100mM NaCl, 5mM MgCl₂, 0.1% Tween) for 10 minutes each time. 500 µl mixture of 9 µl NBT and 7 µl BCIP in AP buffer was added to the embryos in 500 µl AP buffer. The color reaction was stopped, by adding an equal volume (as of the reaction) of 95% ethanol. Embryos were dehydrated in absolute ethanol and rinsed twice in xylene before mounting in permount (Fisher Scientific).

**Dissection and fixation of larvae for *in situ* hybridization**

In order to isolate wing discs larvae were collected from cultures based on their size and developmental stage. Wandering late third instar larvae were removed from surface of the media or from sides of the vials while the early to mid-third instar larvae were pulled out from the media. The dissection was performed in PBT in glass dishes with two pairs of fine number five forceps (Fine Science Tools). Larvae were cut in half by pinching and shearing with forceps. Anterior half was inverted by simultaneously, pushing the mouth hook in with one prong of the forceps and rolling the cuticle from the cut end over the mouth hooks with the other pair of forceps. Inverted larvae were cleaned of other extraneous tissue (gut, esophagus, fat bodies and salivary glands) to better expose the wing discs to the solutions. Cleaned inverted anterior halves of larvae were collected in ice-cold PBT (PBS and 0.1% Tween-20) in eppendorf tubes. After dissecting
for 10 minutes, the ice cold PBT was replaced by 5% w/v formaldehyde made fresh from paraformaldehyde and imaginal discs were fixed for 25 minutes. After end of fixation, the larvae were washed five times with 1ml PBT for a minute or two each time. If not proceeding directly for *in situ* hybridization, the discs were dehydrated gradually into 95% ethanol and stored at -20°C till ready to use. Before using, the dehydrated discs were rehydrated slowly by adding increasing volumes of PBT.

*In situ* hybridization of wing discs

Dissected and fixed larvae were digested 5-8 minutes in 4 µg/ml Proteinase K (Roche). Samples were washed five times: two quick rinses followed by 2-5 minutes three times in PBT. Samples were post-fixed in 5% formaldehyde for 25 minutes at room temperature followed by five washes to remove the fixative. Samples were incubated for 10 minutes in a 1:1 mixture of hybridization solution and PBT. This mixture was replaced by hybridization solution and the samples were nutated for 10 minutes. The hybridization solution was changed and the eppendorf tubes were transferred to a heating block set at 55°C for at least 2 hours. The RNA probes were diluted in the range of 1:100-1:500, denatured by heating at 85°C for 5 minutes and then kept on ice till needed. After pre-hybridization was finished, the hybridization buffer was removed and replaced by the diluted probe and the tube was placed back into the 55°C heating block for 12-18 hours. Probes were removed from the sample and stored at -20°C for reuse. The dissected larvae were rinsed in hybridization solution and then washed four times for 22-30 minutes each time at 55°C. The samples were washed with 75%/25%, 50%/50% and 25%/50% mixture of hybridization solution and PBT respectively, 20 minutes each time at room
temperature, followed by four 15 minute washes in PBT. Samples were then incubated 1-3 hours at room temperature or overnight at 4°C in preabsorbed anti-digoxigenin F\textsubscript{ab} fragments coupled to alkaline phosphatase at a dilution of 1:2000 in PBT. Anti-dig antibody was preabsorbed at 1:10 dilution on posterior halves of dissected larvae that were fixed in the same way as the anterior ends. The preabsorbed antibody was stored at 4°C and used within 3 days. After antibody incubation, samples were rinsed once in PBT and then washed four times, 15 minutes each time in PBT. Samples were washed three times, 5 minutes each time in alkaline phosphatase (AP) buffer. The third wash was sometimes extended to an hour to increase the sensitivity of the staining. Color was developed as described earlier and the reaction was monitored till the reaction was complete. The reaction was terminated by pippeting in an equal volume of 70% ethanol, followed by dehydration into 70% ethanol. The larvae were stored at 4°C till the discs were dissected off and mounted in aquamount (Polysciences Inc.).

**Histological X-gal staining of wing discs**

Larvae were dissected as described before. Dissected larvae were fixed in 1% v/v glutaraldehyde for 15 minutes, washed three times, a minute or two each time. 5-bromo-4-chloro-3-indoyl-\(\beta\)-D-galactosidase (X-gal) staining solution was made to visualize \(\beta\)galactosidase activity as described in (Lehmann and Tautz, 1994). X-gal staining solution consisted of 3.1mM K\textsubscript{4}[Fe\textsuperscript{II}(CN)\textsubscript{6}], 3.1mM K\textsubscript{3}[Fe\textsuperscript{III}(CN)\textsubscript{6}], 0.3% v/v Triton X-100, and 0.2% w/v X-gal in PBS. X-gal solution was made in Dimethyl formamide at 10% w/v dilution. The color reaction was allowed to proceed in dark at 37°C for couple
of hours or overnight. The reaction was stopped by washing with PBT, followed by dehydration by 70% ethanol. The discs were dissected out and mounted in aquamount.

**Antibody staining of wing discs**

Larvae were dissected as described before and fixed in 3.7% formaldehyde (Diluted in PBT from 37% Formaldehyde, Fisher Scientific) for 15 minutes, followed by five washes of 2 minutes each. The discs were blocked with 1% BSA in PBT for an hour. The primary and the secondary antibody were also diluted in 1% BSA. Incubation with primary antibody was carried out at 4°C, overnight. After four washes with PBT, 15 minutes each time, the fluorescence-conjugated secondary antibody was added for 1.5 hours at room temperature. After four 30 minutes washes with PBT, the discs were dissected and mounted in Vectashield and visualized by confocal microscopy.

**Acridine Orange staining of wing discs**

Larvae were dissected in Ringer’s solution (182mM KCl, 46mM NaCl, 3mM CaCl₂·2H₂O and 10mM Tris-Cl, pH 7.2). The tissue was incubated for 5 minutes in 1.6 X 10⁻⁶ M solution of acridine orange (Aldrich) in Ringer’s solution. Immediately before use, 1.6µl of a 1mM stock (1.51mg acridine orange in 5ml absolute ethanol, stored at room temperature, protected from light) in 1ml of Ringer’s. The discs were rinsed briefly in Ringer’s and mounted as quickly as possible in Ringer’s and visualized immediately by confocal microscopy.
Preparation of adult flies for Scanning Electron Microscopy

The flies were dehydrated in ethanol series: 25%; 50%; 75%; 100% and 100% for 24 hours in each solution. 50/50 ethanol/HMDS (Hexamethyldisilazane, Polysciences, Inc.) mixture for 24 hours followed by 100% HMDS for 24 hours. After removal of HMDS, flies were air dried overnight. Flies were mounted and analyzed by scanning electron microscopy.

Generation of Ed intracellular region specific polyclonal antibody

Expression of His-EdIntra protein in E. coli

The C-terminal 600 base pair region of ed was amplified by PCR using the ed cDNA LD2669 as template and the 5’ primer Bam HI-AGTGGCGTCGGCGTCAAT and 3’ primer – HindIII-CTAGACAATAATCTCCGC. The primers were designed such that this EdIntra fragment can be cloned in frame and directionally into the pRSET A vector (Invitrogen). The ligation reaction was transformed into DH5α competent cells. Ampicillin resistant colonies were picked up and screened for the presence of edintra fragment by PCR. Plasmid from one of the positive colonies was purified and sequenced to make sure no mutations were introduced during the PCR amplification process. This clone was transformed into E. coli DE21-pLys S competent cells (Invitrogen). Ampicillin and chloramphenicol resistant colonies were inoculated into 2ml LB (50 g/ml Ampicillin and 100 g/ml Chloramphenicol) and cultured overnight at 37°C. Fresh overnight cultures were diluted 1:100 into LB (Amp+Chlor) and grown for 2 hours. The expression of the protein was induced by adding IPTG to the culture medium at a final concentration of 1mM. The EdIntra protein would have a Histidine-tag at the 5’ end. 1ml
of each of the induced cultures was spun down and the pelleted cells were resuspended in 100 l of 2X SDS-PAGE dye (Sambrook et al., 1989). After boiling the cells for 5min, 10 l of the sample was run on denaturing gel. The expression of the protein was monitored by coomassie blue staining and subsequently by western blot using an antibody against the 6Xhis tag (Qiagen) and compared to uninduced control and also to empty vector (no insert) control. The colonies expressing the highest levels of the fusion protein were used to grow large-scale cultures.

**Large scale expression and purification of 6xHis-EdIntra protein**

10ml of fresh overnight culture was diluted into 1l LB+antibiotics media. Cultures were grown for approximately 2.5 hours till the OD at 600nm reached 0.3-.04. 1ml of the culture was spun down and stored at -80°C. Expression of the fusion protein was induced with IPTG and the cells were cultured for additional 2 hours. 1ml of the culture was spun down and stored at -80°C. Rest of the culture was spun down and frozen at –80°C till purification. Once it was confirmed by coomassie blue staining that protein production had been induced, purification of the protein was carried out.

Purification was carried out under denaturing conditions according to the protocol in QIAexpressionist protocol handbook. Frozen cells were thawed and weighed and resuspended in buffer B (8M Urea, 100 mM NaH2PO4, 10 mM Tris·Cl, pH 8) at 5ml per gram wet weight by gentle vortexing. Lysate was centrifuged at 10,000Xg for 20-30 minutes to pellet cellular debris. Supernatant was added to 50% Ni-NTA slurry (1ml slurry to 4ml cleared lysate). The supernatant was incubated with the Ni-NTA slurry for 60 min at room temperature on a nutator. The lysate–resin mixture was carefully loaded
into an empty column with the bottom cap still attached. The bottom cap was removed and the flow-through was collected for SDS-PAGE analysis. Slurry packed in the column was washed twice with 4 ml buffer C (100 mM NaH2PO4, 10 mM Tris·Cl, 8 M urea, pH 6.3). Wash fraction was kept for SDS-PAGE analysis. The recombinant protein was eluted 4 times with 0.5 ml buffer D (100 mM NaH2PO4, 10 mM Tris·Cl, 8 M urea, pH 5.9), followed by 4 times with 0.5 ml buffer E (100 mM NaH2PO4, 10 mM Tris·Cl, 8 M urea, pH 4.5). Fractions collected and analyzed by SDS-PAGE. Most of the protein eluted in buffer D.

The purified protein at a concentration 10–50 µg/ml was dialyzed back into 100 mM KCl, 2 mM MgCl2, 50 mM HEPES-KOH (pH 7.4) before injecting into guinea pigs (AnimalPharm Services, Healdsburg, CA). A total of 5mg protein was purified for two rounds of immunization into two guinea pigs.

**Generation of Fred specific polyclonal antibody**

A region of *fred* was amplified by PCR using cDNA *SD04816* as template and 5’ primer BamHI- ACCCTATCCGATTGTTCA GGA and 3’ primer HindIII- CTAATACTCACTTCTGCATTG. The resulting 479 bp product represents a region of the extracellular sequence of Fred that is dissimilar to Ed. The cloning and expression of Fred fragment was done similarly to that described for EdIntra. However, the protein was not purified but instead the appropriate sized comassie stained protein band was excised from the gel and send to Cocalico Biologicals, Inc for injection into two rabbits. The production of anti-fred antibody was monitored by western blot analysis in which, the
antibody recognized the 6xHis-fred fragment. However, no expression in embryos was observed.

**Generation of transgenic flies**

**Generation of UAS-EdExt transgenic flies**

EdExtra was generated by PCR using LD2667 as template and the primers, 5’: TAAGATCTCAAAAAATGAGGCGAAAAACTAC and 3’: TATCTAGACTAGCTCTTCTTGCAGATTGC. EdExtra contains the entire Ed extracellular region, transmembrane region and an additional 11 amino acids after the predicted TM domain. Thus, EdExtra contains amino acids 1-1028. EdExtra was inserted into the pUAST transformation vector (Brand and Perrimon, 1993). pUAST-EdExtra was sequenced and transgenic flies were generated via P-element mediated transformation (Spradling and Rubin, 1982). 10 independent transgenic lines were analyzed and subsequently two lines each for insertion on the X, second and third lines were balanced and used for further analysis.

**Generation of UAS-Fred RNAi flies**

The *fred* RNAi construct was generated by using a 638 bp long fragment. This fragment encompasses a part of the putative intracellular region and part of the 3’UTR region of the *fred* cDNA CC3390. This region bears no similarity to *ed* mRNA sequence. The UAS-fred RNAi construct was made as described by Kennerdell and Carthew (2000). Briefly, the fragment was PCR amplified with the primer pairs, forward: *EcoRI* (gaattc)- CACGATCCTGATGAGCAGCTG and reverse: *BSCI* (ggcaagatggcc)-GTTGTTGTTGTTGCTGCTTTGG; and with the primer pairs, forward: *Xhol*(ctgctg)-
CACGATCCTGATGAGCAGCTG and reverse: $SfiI$ (ggccatatctagggc)-GTTGTTGTTGGCTGCTTGG. The two products were digested with $SfiI$ and ligated together. Dimers were digested with $EcoRI$ and $XhoI$, and ligated into the $EcoRI$ and $XhoI$ sites of pUAST (Brand and Perrimon, 1993). The ligation product was transformed into the $E.coli$ SURE strain (Stratagene). Three independent UAS-fred RNAi lines were generated using standard P-element transformation (Spradling and Rubin, 1982). Two of the transgenic lines map to the third chromosome and one to the X chromosome. All three lines produce comparable phenotypes.

**Isolation of fly genomic DNA**

Twenty flies were collected in an eppendorf tube and stored at −20°C shortly to render them unconscious. 500 µl homogenization buffer (0.1M Tris-Cl pH 8.5, 0.1M EDTA, 1% SDS) was added to the eppendorf and the flies were rapidly homogenized with micropestle. Homogenate was incubated at 70°C for 20 min. 70 µl of 8M potassium acetate was added, mixed in by inversion of the tube and stored on ice for 30 min. The tube was spun at maximum speed in a microcentrifuge. The supernatant was transferred to another tube and was spun again if it contained any insoluble material. An equal volume of 50/50 phenol/chloroform was added. After mixing by inverting the tube gently, the tube was spun for 5 min at maximum speed. The upper aqueous phase was transferred to a new tube. The DNA was precipitated by adding 250 µl of isopropanol and spinning the tube for 15 min. The supernatant was discarded and the DNA/RNA pellet was washed with 70% ethanol by adding 100 µl of 70% ethanol and spinning for
5 min. The supernatant was discarded and the pellet for dried shortly and resuspended in 100 µl of 10 mM Tris-Cl, pH 8.

**Sequencing of ed<sup>s</sup> and ed<sup>m1</sup> alleles**

Primers were designed to cover the entire ORF of *ed*. Primers were designed such that only fragments of 500 bp would be generated. High fidelity Taq polymerase was used for amplification. Genomic DNA was isolated from *ed<sup>s</sup>*/ed<sup>s</sup>, *ed<sup>m1</sup>/cnbw* and *cnbw* flies. PCR was performed and the PCR product was analyzed on an agarose gel. Right size PCR products were purified using PCR purification kit (Qiagen) and sequenced with the 5’PCR primer used for the PCR.

**RT-PCR and 5’ RACE**

BLAST search (Altschul et al., 1990) of the *Drosophila* Genome Project Database (Adams et al., 2000) with the *ed* sequence revealed the presence of a gene (*fred*) with a high sequence similarity to *ed*. Fly BLAST against the Berkeley *Drosophila* Genome Project cDNA collection with *fred* yielded four cDNAs representing *fred* transcript(s): GH03216 (Accession no. CG3390), SD04816, SD07424 and SD10817. All these cDNA clones were obtained from Research Genetics and sequenced. SD04816, SD07424 and SD10817 have identical sequence raising the possibility that they represent a single cDNA clone. The ORF of these three cDNA clones is shorter than that of CG3390 due to a stop codon in an apparently unspliced intron. The CG3390 cDNA has no obvious 5'UTR region and thus may be incomplete at the 5' end. The SD cDNAs ORF sequences is closed at the 5' end and has a 5’UTR. Analysis of the genomic region upstream to the 5'UTR, revealed a sequence with a significant similarity to the second IgC2 domain of
ed. The cDNA sequence was extended towards the 5' end by performing reverse transcription reaction using embryonic mRNA as a template and a gene specific primer (GGCATGATGGCGATTTACTCTATA) from the 5' most region common to CG and SD cDNA sequences. Superscript II RT was used for reverse transcription and the reaction was set up according to manufacturer’s protocol (Invitrogen). PCR was performed on the resulting cDNA using a predicted 5’primer (CGCGGAATTTACGATTTGCAA) and nested gene specific 3’ primer CACTCACATTTGACCATTTCG. The cDNA sequence was further extended by 5' RACE (Invitrogen) using the gene specific 3’ primer (AGTGGAGGTCCGGACCCAGAATCC). 5' RACE yielded a cDNA that extends the ORF by 285 bp and has a 377 bp long 5'UTR.

**Probe preparation and array hybridization**

Wing discs were dissected from larvae of the genotype Ap-GAL4; A101 (control; C) and Ap-GAL4; A101:UAS-fredRNAi (experimental; E). Dissected wing discs were lysed in RLT buffer (Qiagen) by pipeting up and down till no wing discs were visible. 100µl RLT buffer was used to lyse 10 wing discs. Lysed wing discs in RLT buffer were stored at -80°C until the next step. Total RNA was extracted from the wing disc lysates using the RNeasy kit (Qiagen). 700 µl of wing disc lyaste was transferred to RNeasy minicolumn. After 15sec centrifugation at 10,000prm, the flow through was discarded. 700µl of buffer RW1 was passed through the column, followed by two washes with 500µl buffer RPE. RNA was eluted in 50 µl of RNase-free water. RNA was quantitated measuring the absorbance at 260nm in a UV spectrophotometer. To ensure significance,
readings used to calculate concentration were above 0.15. An absorbance of 1 unit at 260nm corresponds to 40 µg of RNA per ml of water. 8µg (approximately 200 wandering third instar larvae) of total RNA from each genotype per genechip was processed to produce biotinylated cRNA targets, which were hybridized to *Drosophila* Genechip 1 arrays following standard Affymetrix procedures. Three genechips each were used for both the control and experimental cRNA. The generation of labeled probes and hybridization to the genechips were done at the Microarray Facility at the Cancer Research Center at OSU.

**Microarray data analysis**

Hybridized arrays were scanned using Affymetrix Microarray Suite software as described in manufacturer’s manual. Genechip Analysis suite software was used to create Genechip.chp files for each array. Data from the Genechip.chp files was formatted in Microsoft excel. From the six arrays generated, one control showed aberrant signal that was attributed to uneven hybridization across the chip (Karl Kornacker, Microarray Facility at the Cancer Research Center, pers. communication). In the following analysis we have used results from five (two controls and three experimental) arrays. Signals from the two control chips were averaged (Av. C signal). Similarly, signals from the three experimental chips were averaged (Av. E signal). Av. E signal was substracted from the Av. C signal to arrive at the change in signal between control and experimental chips (Av. C-Av.E). The Av. C-Av.E value was sorted to in descending order. The genes that show a higher signal in the E chips would sort to the end of the list as they would have a negative value. All of the genes that showed an increase in signal (in the E chips versus
the C chip) were copied to another excel sheet. On this sheet the Av C signal was subtracted from the Av. E signal and the resulting data was sorted in descending order to arrive at the list of genes showing increased expression in the E chips vs. the C chips. The fold change was determined by dividing the difference in the control and experimental signals by the control signal. Control genes like 18S rRNA, GAPDH and eIF-4A showed a change of less than 1.3 fold between control and experimental chips. Only the genes that showed 2 fold or higher increase and 1.6 fold or higher decrease are included in the Tables 5.1 and 5.2. Transcripts that showed a decrease in the E chips vs. C chips, were considered only if both the control chips called it present and the C chip signal was above 240. Transcripts that showed an increase in the E chips vs. C chips, were considered only if at least two out of the three experimental chips called it present and the E chip signal was above 240. All the transcripts that were selected by the above mentioned criteria were checked by comparison of individual control chip to experimental chip (six comparisons). Only the transcripts which showed an increase or decrease in at least 4 out of the six comparison were included in the final list (Table 5.1 and 5.2).

**Preparation of template for synthesizing RNA probes**

For the synthesis of dig-labeled RNA, the templates used were either PCR products with a T7 promoter introduced on the 3’ side by PCR primer, or PCR products cloned into pCRII-Topo vector (Invitrogen) or cDNA clones obtained from various labs.

Primer sequence for generation of PCR products:

CG5164
Forward: CATGTCGAGCTCTGGAAATTG
Reverse: TTTCACTTTGTCGCCAGCTT
CG3074
Forward: AAATCTCCACCGACACGACT
Reverse: CAAATAGGCCTGAGGGTTGT

CG15784
Forward: ATGAGCGAGTTGTCCAGACT
Reverse: TAATACGACTCACTATAGGGCTAATCACGTCATGTCCATG

CG4914
Forward: ATGAGCGAGTTGTCCAGACT
Reverse: TAATACGACTCACTATAGGGCTAATCACGTCATGTCCATG

HLHmbeta
Forward: TGACACAGAGTCTCCGAG
Reverse: TAATACGACTCACTATAGGGGAGCAGCCATCTCTTCTATAGCCG

Mirror
Forward: GTTCCACGAGACGACTGAGGAACCGCTGATGATTATCA
Reverse: TAATACGACTCACTATAGGGGAGCAGCCATCTCTTCTATAGCCG

Pannier
Forward: ATCGGCACACCAACAATGCT
Reverse: TAATACGACTCACTATAGGGCATTCCAGGCAGCTCTTAAAC

CG12505
Forward: TACTCAGATGGCAATCGGCGT
Reverse: TAATACGACTCACTATAGGGCATTCCAGGCAGCTCTTAAAC

CG10916
Forward: TGGAACAGAAGAATAGAGGCA
Reverse: TAATACGACTCACTATAGGGGACAGGATCATAAAATAGGCA

CG3734
Forward: GGCAATATTATATTTACTGGCTTAA
Reverse: TAATACGACTCACTATAGGGGATGCGGGTTTCTTCCG

Tsp42Ed/CG12846
Forward: ATCTCTACCAGCAGCAAC
Reverse: TAATACGACTCACTATAGGGCAGTGATGTTGAATAATGAAGCA
T7 promoter sequence is in italics. Apterous cDNA was kindly provided by S. Cohen. Homothorax cDNA was from A. Salzberg. Bearded cDNA was provided by M. Posakony.

Fly stocks

The following *Drosophila* strains were used: UAS-fred RNAi (this study), pnr<sup>MD237</sup>-GAL4 (Heitzler et al., 1996a), ap- GAL4 (Calleja et al., 1996), neutralized (neu)-lacZ A101 (Bellen et al., 1989), Eq- GAL4 (Pi et al., 2001), GMR-GAL4 (Freeman, 1996), UAS-N (Doherty et al., 1997), Su (H)<sup>AR9</sup> (Schweisguth and Posakony, 1992), UAS-H (Nagel et al., 2000), UAS-Su (H) (gift from L. Seugnet and M. Haenlin via S. Bray), UAS-E (spl) m7 (Ligoxygakis et al., 1999), ed<sup>2B8</sup>, ed<sup>3C2</sup> (Bier et al., 1989), ed<sup>K01102</sup> are P-elements insertions in the ed locus. ed<sup>nl</sup> and ed<sup>a</sup> are EMS induced ed mutant alleles (Ahmed et al., 2003), Gap1<sup>B2</sup> (Chou et al., 1993), pnt<sup>88</sup> (Brunner et al., 1994), SRV-lacZ (Culi and Modolell, 1998), and Df(1)sc10-1 (Hinz et al., 1994; Lindsley and Zimm, 1992). Df(1)sc10-1, neur-lacZ A101, UAS-lacZ, Gap1<sup>B2</sup>, pnt<sup>88</sup>, Df(2L)ed1, Df(2L)M24F11, ed<sup>K01102</sup>, E(spl)<sup>8D06</sup>/TM3,Sb, T80 GAL4 and GMR-GAL4 were obtained from the Bloomington *Drosophila* Stock Center. 69B GAL4 was kindly provided by A. Garcia-Belido. For genetic interaction studies the following stocks were used: UAS-N<sup>act</sup>: ed<sup>as</sup>/UAS-N<sup>act</sup>: ed<sup>as</sup> (the UAS-N<sup>act</sup>: ed<sup>as</sup> chromosome was generated by standard recombination), ed<sup>2B8</sup>/P(actin5C-lacZ)Cyo; Kr-GAL4/ P(actin5C-lacZ) TM3Sb, UAS-N<sup>act</sup>/Cyo (E. Giniger and B. Yedvobnick), Df<sup>1al</sup>/TM3,Sb (Vaessin and Campos-Ortega, 1987), E(spl)<sup>8D06</sup>/TM3Sb (Klamt et al., 1989; Knust et al., 1987), UAS-EdExt (this
study), \( pnr^{MD237} \)-GAL4 (Heitler et al., 1996b), Eq-GAL4 (gift from C. T. Chein), UAS-
\( E(spl)m7 \) (Ligoxygakis et al., 1999) and UAS-DI (Doherty et al., 1996).

**Genetic crosses**

\( UAS-N^{act}: \) \( ed^{ts}/ UAS-N^{act}: \) \( ed^{ts} \) fly lines were isolated by screening for recombination in females of the genotype \( UAS-N^{act}/ ed^{ts} \). Screening strategy: These females were crossed to Sco/CyO males. CyO male progeny of this cross were tested in single male cross to 1) Kr-GAL4 flies; 2) \( ed^{2B8}/ CyO; \) 3) Sco/CyO. If the male carried \( UAS-N^{act}, \) no CyO\(^+\) and Sb\(^+\) flies would emerge from the first cross. If the male carried \( ed^{ts}, \) no CyO\(^+\) flies would emerge from the second cross, cultured at 29\( ^\circ \)C. Once it was determined that the male has both \( UAS-N^{act} \) and \( ed^{ts} \) on the same chromosome, \( UAS-N^{act}: \) \( ed^{ts}/ \) CyO stock was established. In the next generation, \( UAS-N^{act}: \) \( ed^{ts}/ UAS-N^{act}: \) \( ed^{ts} \) flies were selected to establish a homozygous stock. \( ed^{2B8}/P(\text{actin5C-lacZ})\text{Cyo}; \) Kr-GAL4/\( P(\text{actin5C-lacZ})\text{TM3Sb} \) fly lines were generated by using the doubly marked strain \( T(2;3)/P(\text{actin5C-lacZ})\text{Cyo}; \) \( P(\text{actin5C-lacZ})\text{TM3Sb} \). The cross was carried out at 29\( ^\circ \)C using \( UAS-N^{act}: \) \( ed^{ts}/ UAS-N^{act}: \) \( ed^{ts}; +/+ \) males and \( ed^{2B8}/P(\text{actin5C-lacZ})\text{Cyo}; \) Kr-GAL4/\( P(\text{actin5C-lacZ})\text{TM3Sb} \) virgins. Embryos of the genotype \( UAS-N^{act}: \) \( ed^{ts}/ed^{2B8}; \) \( Kr-GAL4/+ \) were identified by lack of lac-Z expression detected by staining with anti-\( \beta \)galactosidase antibody (Promega, Mouse anti-\( \beta \)galactosidase).

For wing disc dissection: females homozygous for \( UAS-fred\, RNAi \) (on X or third chromosome) were crossed to \( A101: \) \( pnr-GAL4/TM6 \) \( Tb \) (recombined) flies and \( Tb^{+} \) larvae were dissected; \( UAS-fred\, RNAi: A101/TM6 \) \( Tb \) (recombined) were crossed to \( ap- \)
GAL4/Gla-Bc; wg-lacZ and Bc+ and Tb+ larvae were dissected to determine the effect of fred RNAi. Flies were raised at 25°C, unless otherwise mentioned.
CHAPTER 3

echinoid mutants exhibit neurogenic phenotypes and interact synergistically with Notch signaling pathway

INTRODUCTION

$ed^{2B8}$ was isolated in an enhancer-trap screen (Bier et al., 1989). The P-element insertion was mapped to the 24D3-4 region on the left arm of the second chromosome by in-situ hybridization. The embryonic recessive lethality was mapped to this region using the overlapping deficiencies: $Df(2L)ed1$ (24A3-4; 24D3-4) and $Df(2L)M24F11$ (24D3-4; 25A2-3). To test whether the phenotype of $ed^{2B8}$ homozygous embryos is associated with the P-element insertion in the $ed$ locus, the P-element was remobilized. A complete reversion of the mutant phenotype was observed (Vaessin, unpublished). In situ hybridization and antibody labeling with anti-Ed antisera does not show detectable levels of $ed$ gene products in $ed^{2B8}$ homozygous mutant post-blastoderm embryos (Ahmed, 1999), indicating that $ed^{2B8}$ represents a strong hypomorphic or amorphic $ed$ allele. Additional lethal P-element insertion lines mapping to the 24D region were obtained from a collection of recessive lethal P-element insertion lines maintained at Bloomington Stock Center. The pupal lethal P-element insertion line, $l(2)k01102$ carries a P-element in the first intron of the $ed$ transcription unit and was
identified as an ed allele based on non-complementation of \(ed^{2B8}\) (Bai et al., 2001). Two additional recessive embryonic lethal ed mutations were isolated. \(ed^{3C2}\) originated from a P-element screen for recessive embryonic lethals (Bier et al., 1989). \(ed^{m1}\) was isolated as an EMS induced mutation, which is embryonic lethal in transheterozygosity with \(ed^{2B8}\). A temperature sensitive ed allele \(ed^s\) was also isolated from the EMS screen. \(ed^s\) is embryonic lethal in transheterozygosity with \(ed^{2B8}\) at 29°C but is viable in homozygosity or in transheterozygosity with \(ed^{2B8}\) and other ed alleles at 25°C (Ahmed et al., 2003).

Immunostaining of homozygous (\(ed^{2B8}\), \(ed^{3C2}\) and \(ed^{m1}\)) or transheterozygous ed mutant embryos (\(Df(2L)ed1/ed^{2B8}\), \(ed^{2B8}/ed^{m1}\), \(ed^{2B8}/ed^s\), and \(ed^{2B8}/ed^{3C2}\) ) with anti-HRP antibody, which recognizes all neuronal cells of the CNS and PNS (Jan and Jan, 1982), reveals hyperplasia of the CNS. The hyperplasia ranges from mild “bulges” in the CNS to an extensive increase in the size of the entire CNS (data not shown). An intermediate phenotype is shown in Figure 3. 1 (Figure. 3. 1A, B and data not shown). Analysis of ed mutant embryos with antibodies against a neural precursor marker, Deadpan (Bier et al., 1992), shows that the increase in the number of neurons is preceded by an increase in the number of neural precursors (Figure 3. 1E, F). The enlargement of the CNS is accompanied by a parallel loss of epithelium, manifested as loss of cuticle, from the ventral and procephalic regions of the embryo. Similar to the variable neural hyperpalsia exhibited by ed mutant embryos, the cuticular defect range from a fusion of denticle belts to a complete absence of cuticle from the procephalic, ventral and ventrolateral regions (Data not shown and Figure 3. 1C, D) (Ahmed et al., 2003). The ed mutant phenotype is reminiscent of the phenotypes displayed by loss of function mutations of neurogenic genes that are part of the Notch signaling pathway (Lehmann et al., 1983).
In order to further define the role of Ed in embryonic and adult neurogenesis, I have extended the study of Ed done by A. Ahmed. Results of my study, together with A. Ahmed’s data support the conclusion that Ed is required to neurogenesis during both embryonic and adult stages and it synergizes with the Notch signaling pathway in this process.

RESULTS

**Ed shows a dynamic expression pattern during embryogenesis**

*ed* has previously been shown to be expressed uniformly in the developing larval wing and eye discs (Bai et al., 2001). In contrast, our analysis of both *ed* RNA and protein expression during embryogenesis revealed a dynamic spatial and temporal expression pattern of *ed* gene products. At stage 5, *ed* is expressed in all cells except the pole cells (data not shown). During stage 9, both, *ed* RNA and protein expression is eliminated from the delaminated neuroblasts, while it is still present in the mesodermal and ectodermal cells (Figure 3. 2A, C). In addition, subcellular localization of the *ed* transcript becomes evident during this stage. Thus, *ed* RNA appears to be highly concentrated in the basal region of the ectodermal cells, while no or only low levels of *ed* RNA is detectable in the apical portions of the ectodermal cells (Figure 3. 2B). A similar localization of *ed* protein can be observed during this stage (Figure 3. 2D). During stage 11 *ed* expression becomes more dynamic and a reduction of *ed* expression in the ventral neurogenic region becomes evident. In parallel, high levels of Ed expression is visible in the tracheal pits (Figure 3. 2E). At late stage 11/early stage 12, *ed* expression in the ventral neurogenic region is terminated. Later in development,
ed expression is detected in a variety of developing tissues. These include epidermis, muscle, the hindgut and foregut (Figure 3. 2H and data not shown). In addition, ed expression is evident in a subset of cells in the CNS (Figure 3. 2F). At stage 16 ed protein becomes predominantly localized to axons and appears particularly concentrated in the posterior commissures (Figure 3. 2G).

**Embryonic lethal alleles of ed**

The P-element associated mutation in ed^2B8 results in substantial reduction in expression levels of ed. Although this mutation shows that Ed is necessary for embryonic development, it does not offer any clues to the amino acids or domain/s of Ed that are essential for its function. For the ed^ts and ed^m1 mutations however, the Ed protein is synthesized but it is not functional. The mutation associated with these alleles would thus be informative about the essential amino acids of Ed which when mutated result in a non-functional/unstable protein. To determine the mutation in these two alleles, I extracted genomic DNA from ed^ts/ed^ts, ed^m1/cn bw and cn bw flies. The ed ORF was sequenced (materials and methods Chapter 2) and compared to the ed ORF of cnbw flies. In case of ed^ts, I looked for difference in DNA sequence as well as the amino acid sequence between ed^ts flies and cnbw flies. For, ed^m1/cnbw flies I looked for both the differences in DNA sequence from the cnbw flies as well as calls for an ambiguous base (i.e. an N). Since the DNA from ed^m1/cnbw is 1:1 mutant:wt, I have to look for a polymorphism. Sometimes, the sequence analysis software does not call an N. So I also visually scanned the electropherogram for ambiguous-looking peaks.
The mutation associated with \( ed^6 \) was mapped to a single base change, G\(^{1834} \) to A (data not shown). This results in the replacement of the conserved Asp\(^{612} \) residue in the IgC2 domain VI with an Asn residue. The molecular basis of \( ed^{m1} \) has not been determined yet. Sequencing of the \( ed^{m1} \) allele ORF did not reveal a mutation. It is possible that the polymorphism between the wt and \( ed^{m1} \) allele was not called out as an N but as the wt base and I missed the change in the peak size when I scanned the electropherogram.

**Ed has multiple functions during *Drosophila* development**

*ed* has previously been shown to be essential for proper eye and wing formation (Bai et al., 2001; Islam et al., 2003; Rawlins et al., 2003a; Spencer and Cagan, 2003). To further examine *ed* function during postembryonic development, the phenotype of several transheterozygous viable combinations of *ed* alleles was analyzed. Flies transheterozygous for \( ed^{k01102} \) and \( ed^{m1} \) (\( ed^{k01102}/ed^{m1} \)) generally die as pupae, but escaper flies can be isolated from low-density cultures and their adult phenotype examined (Ahmed, 1999). Flies transheterozygous for \( ed^6/ed^{m1} \) or \( ed^6/ed^{2B8} \), shifted to the nonpermissive temperature of 29\(^\circ\)C for three days at the end of larval stage 2, and then shifted back to 18\(^\circ\)C, can also survive to adulthood. These flies show defects in the wings, legs, sensory bristles and eye. Frequently, transheterozygous *ed* mutant flies show extra wing vein material, irregular thickening of wing vein II and notching at the distal tip (Figure 3. 3A, B and data not shown). The leg phenotypes visible in transheterozygous flies were generally mild. Thus, the legs of \( ed^{k01102}/ed^{m1} \) males had proper number of segments, but the sex combs were misarranged (Figure 3. 3C, D). The sensory bristles,
macrochaetae and microchaetae, are arranged in a stereotypical pattern on the adult dorsal thorax (Jan and Jan, 1993b). $ed^{ts}/ed^{m1}$ or $ed^{ts}/ed^{2B8}$ flies that were shifted to the nonpermissive temperature as third instar larvae (see above) show extra macrochaetae. These extra macrochaetae were generally found next to macrochaeta normally present in the wild type (Figure 3.3 E, F). The microchaetae were also increased in density. In addition, $ed^{ts}/ed^{m1}$ or $ed^{ts}/ed^{2B8}$ flies and the $ed^{k01102}/ed^{m1}$ showed a rough eye phenotype (not shown), similar to the eye phenotype described previously (Bai et al., 2001).

**Overexpression of the extracellular region of Ed results in a dominant negative phenotype**

The extracellular region of Ed has seven IgC2 type domains and two-fibronectin type III repeats. Transgenic fly lines, expressing a truncated Ed protein ($Ed^{Ext}$) containing the entire extracellular and TM region, but lacking most of the intracellular region (except for the first 11 amino acids of the intracellular region), were generated. $Ed^{Ext}$ was expressed in wing discs of otherwise wild type flies using various GAL4 drivers. Ectopic expression of $Ed^{Ext}$ in the wing disc using the either the $T80\ GAL4$ or the $69B\ GAL4$ driver lines resulted in extra wing vein material at the distal end of wing vein II and rare (10 percent, n=30 wings) notching of the distal wing tip (Figure 3. 4B,C and data not shown). These phenotypes are similar to those seen in the adult hypomorphic $ed$ mutant flies. In addition, and similar to adult flies carrying hypomorphic $ed$ alleles, these animals also show extra sensory bristles (macrochaetae and microchaetae) (Figure 3. 4E and data not shown). Extra macrochaetae were always found in close proximity to a normally positioned bristle. The extra sensory bristles phenotype was more prominent when a stronger wing disc driver, $pnr\-GAL4$ was
used. *pnr-GAL4* mediates expression in the mesothorax region (Heitzler et al., 1996b). Expression of Ed\textsuperscript{Ext} in this region resulted in an increase in the number of macrochaeta. This was especially evident in the scutellar region. Extra bristle specification was accompanied by an apparent loss of epidermal tissue, resulting in a smaller scutellum (Figure 3. 4F). The loss of epidermal tissue is much more severe than what would result from the inappropriate SOP specification. At present it is not clear whether Ed has a role in epidermogenesis of whether this phenotype is a non-specific effect of Ed\textsuperscript{Ext} overexpression. In addition, microchaetae were duplicated or quadrupled and missing their respective sockets, or missing completely (Figure 3. 4G). The phenotypes observed upon overexpression of the Ed\textsuperscript{Ext}, are qualitatively similar to those observed with various transheterozygous combinations of *ed* mutant alleles. These observations suggest that the Ed\textsuperscript{Ext} construct behaves as a dominant negative protein.

**ed interacts with genes of the Notch signaling pathway**

The neurogenic phenotype of homozygous *ed* mutant embryos as well as the extra sensory bristle phenotype of *ed* transheterozygous adults suggests that *ed* gene function, similar to the function of N signaling pathway genes, may be required to suppress neural fate. We, therefore, performed genetic interaction tests to investigate the possibility of functional interactions and /or epistatic relationship between *ed* and genes of the N signaling pathway.

Activation of the N signaling pathway results in suppression of neuronal cell fate (Bray, 1998; Greenwald, 1998). Overexpression of the intracellular region of N (N\textsuperscript{act}) results in a dominant activated phenotype (Fortini et al., 1993; Rebay et al., 1993). Accordingly, when N\textsuperscript{act} is expressed in embryos using the *Kr-GAL4* driver, an almost
complete loss of neurons in parasegments 4-6 (Kr expression domain) is detected (Lieber et al., 1993); and Figure 3. 5C and G). In contrast ed^{ts}/ed^{2B8} transheterozygous embryos, grown at the restrictive temperature of 29°C, show a phenotype opposite to that caused by ectopic N^{act} expression. Here, hyperplasia of the CNS with local bulging of the ventral nerve cord is evident (Figure 3. 5B and F). Reduced ed activity results in a strong suppression of the N^{act} overexpression phenotype, as is evident in the CNS of UAS-N^{act}; ed^{ts}/ed^{2B8}; Kr-GAL4/+ embryos (Figure 3. 5D and H). Hence, reduction of ed activity can compensate for ectopic N signaling pathway activity.

To test for genetic interactions between ed and other genes of the N signaling pathway A. Ahmed performed dosage sensitive interaction assays. For this purpose, a homozygous viable Dl mutation, Dl^{via1} (Vaessin and Campos-Ortega, 1987) and E(spl)^{8D06}, a deletion of the E(spl) complex (Knust et al., 1987), were used. Flies heterozygous for Dl^{via1} (+/+; Dl^{via1} /+) have wings indistinguishable from the wild type. ed^{ts}/ed^{m1}; +/- flies, grown at 25°C have wings with irregular thickenings in wing vein II (Figure 3. 5J). In contrast, the wings of ed^{ts}/ed^{m1}; Dl^{via1} /+ flies, grown at 25°C, display significantly increased irregular thickenings of wing vein II. In addition, extra wing vein material is evident in the posterior cell of the wing (Figure 3. 5K). Wings of +/-; E(spl)^{8D06}/+ flies, raised at 29°C, are generally wild type, but may display with low penetrance (< 20 %), extra vein material in the posterior cell (Figure 3. 5M). Flies heterozygous for ed^{ts} (ed^{ts}+/; +/-), raised at 29°C, have morphologically normal wings (Figure 3. 5L). The wings of ed^{ts}/+; E(spl)^{8D06}/+ flies, raised at 29°C, showed an enhanced phenotype compared to +/-; E(spl)^{8D06}/+ flies: the extra vein material is expanded in ed^{ts}/+; E(spl)^{8D06}/+ flies and is visible with full penetrance (Figure 3. 5N).
Thus synergistic interactions can be observed between \textit{ed} and \textit{Dl}, as well as \textit{ed} and the \textit{E(spl)-C}.

I also analyzed the ability of the dominant negative \textit{Ed}^{\text{Ext}} protein to functionally interact with \textit{N} signaling pathway genes. In these experiments \textit{Ed}^{\text{Ext}} was ectopically expressed using the \textit{Eq-GAL4} driver. \textit{Eq-GAL4} mediates expression in the anterior region of the presumptive notum in the wing disc, with a stronger expression in the anterior midline region (Pi et al., 2001). \textit{UAS-Ed}^{\text{Ext}} /+; \textit{Eq-GAL4}/+ flies exhibit a slight increase in the number of microchaetae (Figure 3.6B). Overexpression of full-length Delta has been shown to cause an increase in the number of sensory organs (Doherty et al., 1997); Figure 3.6C. It has been argued that this is due to the ability of \textit{Dl} to autonomously inhibit \textit{N} signal reception in \textit{Dl} expressing cells. Hence, when all cells of the proneural clusters express high levels of \textit{Dl}, \textit{N} signal reception would be inhibited, causing an increase in the number of sensory organs (Doherty et al., 1996; Doherty et al., 1997). Simultaneous overexpression of \textit{Dl} and \textit{Ed}^{\text{Ext}} using the \textit{Eq-GAL4} driver resulted in a dramatic increase in the number of microchaetae that exceeds the additive combination of the respective phenotypes (Figure 3.6D). Hence, dominant negative \textit{Ed}^{\text{Ext}} causes increased reduction in \textit{N} signaling pathway mediated lateral inhibition caused by ectopic expression of \textit{Dl}. Accordingly, increase of the activity of the \textit{N} signaling pathway should suppresses the phenotype caused by \textit{Ed}^{\text{Ext}} overexpression. Flies overexpressing \textit{Ed}^{\text{Ext}} in the \textit{pnr} expression domain (\textit{UAS-Ed}^{\text{Ext}} /+; \textit{pnr-GAL4}/+) show extra sensory organs (Figures 3.4F and 3.6F). Ectopic expression of \textit{E(spl)m7}, a downstream target of activated \textit{N} signaling pathway, suppresses sensory organ specification resulting in the absence of bristles (Ligoxygakis et al., 1999). Flies expressing ectopic \textit{Ed}^{\text{Ext}} and
E(spl)m7 simultaneously are indistinguishable from those expressing only E(spl)m7 ectopically (Figure 3. 6H), implying that increasing the activity of the N signaling pathway can compensate for reduced Ed activity.

**DISCUSSION**

**Echinoid is required for the restriction of neuronal development**

Using the embryonic lethal alleles of *ed*, we have shown that *ed* is essential for normal embryonic neurogenesis. The phenotypes associated with loss of function mutations of *ed* have characteristic hallmarks of mutant phenotypes shown by neurogenic genes such as *N, Dl* or the *E(spl)* gene complex, namely hyperplasia of the nervous system and corresponding loss of epidermal structures. Similarly, reduction of *ed* function during sensory organ formation in the wing disc results in specification of extra sensory organs. This phenotype implies that *ed* is also required for the process of lateral inhibition in the proneural clusters during adult sensory organ development. A main difference between the mutant phenotypes of neurogenic genes and of *ed* alleles lies in the severity of the observed hyperplasia of the embryonic CNS. Embryos homozygous for apparently amorphic *ed* alleles show a less extensive neural hyperplasia than that caused by loss of genes such as *N* or *Dl* and resemble embryos homozygous/hemizygous for hypomorphic alleles of other neurogenic genes. Strong maternal effects contribute to weak phenotypes of various amorphic mutant alleles of neurogenic genes like *N, mam* or *groucho*. Indeed, maternal *ed* transcripts can be readily detected in Northern blot analysis of 0-2 hour old embryos ((Ahmed, 1999) and data not shown). However, it remains to be
determined if a maternal contribution can account for the relatively weak neural hyperplasia exhibited by ed mutant embryos.

*ed* RNA and protein expression during early neurogenesis indicates that *ed* gene products become restricted to the neuroectodermal cell layer, while no *ed* products are detectable in the delaminated neuroblasts. The dynamics of *ed* RNA and protein distribution during neuroblast delamination implies that *ed* function might be required in cells that remain in the ectodermal cell layer. In such a scenario, similar to N, *ed* function would be required in the cells receiving the lateral inhibitory signal. However, it should be noted that at the time when the differential distribution of *ed* RNA and protein becomes detectable, the neuroblast segregation has already been initiated.

Ed expression during embryogenesis is dynamic and seen in many developing organ systems. The widespread expression of *ed* indicates that *ed* might be required for the development of multiple organs. Indeed, analysis of the trachea and muscles in *ed* mutant embryos reveals defects in the proper formation of these organ system (data not shown). The requirement of *ed* for normal development of multiple tissues is not limited to embryogenesis. Adult flies with reduced *ed* activity, show defects in leg, wing and eye development (Bai et al., 2001; Islam et al., 2003). A similar widespread expression and requirement in multiple organs has also been observed for the Notch signaling pathway during *Drosophila* development (Artavanis-Tsakonas et al., 1999).

**The ectopically expressed extracellular domain of Ed has dominant negative activity**

Ed protein missing its intracellular region interferes with the process of lateral inhibition, as overexpression of Ed\textsuperscript{Ext} in the developing wing disc results in an increase in
the number of macrochaetae and microchaetae. Additional phenotypes include the irregular thickening of wing vein II and infrequent notching of the wing margin. These phenotypes are similar to those seen upon reduced ed function. Thus, ectopic expression of Ed\textsuperscript{Ext} interferes with the function of endogenous Ed. A dominant negative activity of the extracellular portion is not unusual for receptors that bind to ligands and then transduce a signal intracellularly (Rebay et al., 1993). Thus it is possible that the Ed\textsuperscript{Ext} competes with the wild type Ed for a limited amount of ligand. Since Ed\textsuperscript{Ext} is missing its intracellular region, its binding to the ligand may have no functional consequence other than limiting the amount of available ligand. The ability of the extracellular domain to act as a dominant negative molecule and the observation that the temperature sensitive allele of ed has a mutation associated with Ig C2 domain VI implies that the interaction of the extracellular domain with a putative ligand is an essential component of Ed function.

Neuroglian (both Nrg\textsuperscript{180}, the long isoform and Nrg\textsuperscript{167}, the short isoform) has recently been identified as an activating ligand for the antagonistic effect of Ed on the EGFR pathway in the eye disc (Islam et al., 2003). Both isoforms are expressed in the wing disc, Nrg\textsuperscript{180} is neuron specific, whereas, Nrg\textsuperscript{167} is expressed rather uniformly (Hortsch et al., 1990). It has yet to be determined whether Nrg also functions as a ligand for Ed during sensory organ development.

Ed has been shown to act as a homophilic cell adhesion molecule (our unpublished results and Islam et al., 2003). In the eye disc, it has been shown that the Nrg mediated heterophilic activity of Ed in repressing EGFR signaling pathway is redundant with the homophilic activity of Ed (Islam et al., 2003). Thus it is possible that the
dominant negative construct interferes with Ed activity by competing for homophilic binding.

**Ed interacts synergistically with the N signaling pathway**

Ectopic expression of an activated form of N results in suppression of neuronal specification (Lieber et al., 1993; Rebay et al., 1993; Struhl et al., 1993). In contrast, reduced ed gene activity results in increased specification of neurons. Ectopic expression of N^{act} in ed^{2B8/ed} embryos results in a near wild type nervous system. The observation of compensating, as opposed to an epistatic phenotype, does not support the formulation of straightforward epistatic relation between ed and N gene function. Rather, while both, wild type N and ed, have a similar antineurogenic function during neurogenesis, they might be acting in functionally synergistic, yet possibly parallel regulatory pathways.

The observation of dosage sensitive interactions between mutations in two genes can also be indicative of closely related roles. We have observed dosage sensitive interactions between ed and Dl and ed and E(spl). The mild wing phenotype exhibited by ed^{m1/ed} flies raised at 25°C is enhanced by loss of one copy of Dl. Similarly the wing phenotype of E(spl)^{8D06/+} flies is enhanced by reduction of Ed activity. These observations imply that, in the wing disc also, the N signaling pathway and ed are acting synergistically.

Genetic interaction between ed and the N signaling pathway is also observed during the development of the adult PNS. Ectopic expression of Ed^{Ext} results in specification of extra macrochaetae and microchaetae. Overexpression of Dl results in an increase in the number of sensory bristles (Doherty et al., 1997). Simultaneous ectopic
expression of $Ed^{Ext}$ and $Dl$ has a phenotype much stronger than what would be the result of additive combination of the individual phenotypes. The $Ed^{Ext}$ protein interferes with the activity of endogenous $Ed$ and the decrease in $Ed$ activity increases the neurogenic phenotype caused by $Dl$ overexpression. These observations imply that $Ed$ acts in concert with $Dl$. An epistatic interaction was also observed with $E(spl)m7$. Ectopic expression of $E(spl)m7$ completely suppressed the extra bristles phenotype obtained upon $Ed^{Ext}$ expression. The complete suppression of the dominant negative phenotype would imply that $E(spl)m7$ functions downstream to the $ed$. However, it is possible that this suppression is a result of the strong antineurogenic activity of $E(spl)m7$. Although it is presently not clear whether $ed$ and the genes of the $N$ signaling pathway function in the same or parallel pathway, our observations establish that $ed$ and the $N$ signaling pathway genes act synergistically in both, embryonic and postembryonic development.

**Ed, Fred and the N and EGFR signaling pathway**

$Ed$ has previously been shown to be a negative regulator of the EGFR pathway. $ed$ mutations enhance the rough eye phenotype of $Elp^{B1}$, a gain-of-function EGFR allele and $ed$ genetically interacts with several components of the EGFR pathway during eye development. As a consequence, $ed$ mutant phenotypes include the generation of extra photoreceptor and cone cells (Bai et al., 2001). We have shown that $ed$ mutations result in neural hyperplasia in the embryo and the formation of extra sensory organs on the notum of adult flies, and that $ed$, in these processes, interacts synergistically with the $N$ signaling pathway. We have recently described an $Ed$ paralog, Fred and have shown that Fred is required to suppress SOP specification and is required for proper eye
development (Chandra et al., 2003). Similar to ed, fred interacts synergistically with the N signaling pathway during SOP specification and also during eye development. fred also interacts with EGFR pathway. Furthermore, ed and fred show a dosage sensitive interaction during eye development, indicating that the two genes function in close concert (Chandra et al., 2003). Hence, ed and fred define a subgroup of two closely related IgC2 type transmembrane proteins that interact synergistically with the N signaling pathway and antagonistically with the EGFR pathway.

**Putative role of a cell adhesion protein in neuronal cell specification**

Our data suggest a role of Ed in the processes of cell-cell communications that result in the selection of the future neural precursor from a group of cells with an equivalent potential to develop into neural precursors. In this process, we have shown that ed functions synergistically with the N signaling pathway. Although it is possible that Ed might be acting at the level of cell-cell communication via trans-homophilic or heterophilic interaction to limit neural precursor specification, however, keeping in mind, its cell-cell adhesion function, it can be imagined that Ed is also involved in the execution of the information relayed by the cell-cell communication that occurs between the future neural precursor and the future epidermal precursors. Thus it is possible that the future neuroblast downregulates adhesion, by downregulating Ed and delaminate from the epidermal precursors and the future epidermoblasts do the opposite. In the absence of Ed, adhesion might be compromised such that more cells delamate from the neuroectoderm and go on to form neurons. Thus, Ed might be functioning at the level of cell-cell
communication and/or at the level of coordinating cell-cell signaling with morphogenesis.
Figure 3.1: Embryonic phenotypes associated with ed mutations. Ventral views of wild-type (A) and ed<sup>2B8</sup> / ed<sup>2B8</sup> (B) embryos stained with anti-HRP antibody. ed<sup>2B8</sup> homozygous embryos exhibit a hyperplasia of the CNS. This hyperplasia is accompanied by loss of epidermal structures. (C-F) are lateral views of the embryos. Cuticle preparations of wild-type (C) and ed<sup>2B8</sup> / ed<sup>2B8</sup> (D) embryos. Asterisks in (C) mark denticle belts, characteristic structures of the ventral cuticle. ed<sup>2B8</sup> homozygous embryos show an extensive loss of ventral and procephalic cuticle. (E) and (F) are lateral views of wild-type (E) and ed<sup>2B8</sup> homozygous (F) embryos labeled with anti-Deadpan antibody. Arrowheads in (F) mark the ectopic neuroblasts present in ed mutant embryos.
Figure 3.1: Embryonic phenotypes associated with ed mutations
Figure 3. 2: *ed* expression pattern during embryogenesis. *ed* RNA (A, B) and protein (C-H) expression in wild type embryos. In stage 9 embryos (A-D), differential expression of *ed* is evident. While *ed* is expressed in the neuroectodermal (NE) and mesodermal (ME) cell layer, no expression is detected in the delaminated neuroblasts. (B) and (D) show enlarged lateral views of the ventral regions of embryos in (A) and (C), respectively. The NE and ME cell layer are indicated and the bracket spans the layer where delaminated neuroblasts are located. (E) By stage 11, Ed expression ceases in the ventral neurogenic region and becomes restricted to the tracheal pits, marked by the asterisk, and the epidermis. At late stage 13, Ed can be detected in subset of cells in the developing CNS and also in the epidermis (F). During stage 14, Ed expression is limited to the anterior and posterior epidermal stripes (G). At stage 16, Ed is highly localized to the axons with a higher concentration in the posterior commissures (H). A and P stand for anterior and posterior commissure respectively.
Figure 3.2: *ed* expression pattern during embryogenesis
Figure 3. Adult phenotypes associated with ed mutations. (A) Wings of wild type and (B) ed^{2B8}/ed^{ts} flies. ed^{2B8}/ed^{ts} flies shifted to 29°C for three days at end of larval stage 2 show wings with irregular thickening of wing vein II and/or slight notching (indicated by the bracket in B) of the distal margin. In addition, extra macrochaetae and an increased density of microchaetae are evident on the thorax of ed^{2B8}/ed^{ts} flies (F), compared to the wild type (E). Arrowheads point to ectopic bristles. Foreleg of wild type (C) and ed^{k01102}/ed^{m1} (D) males. The sex combs are misformed.
Figure 3.3: Adult phenotypes associated with ed mutations.
Figure 3. 4: Ed$^{\text{Ext}}$ has dominant negative activity. (A) Control wing of $UAS-Ed^{\text{Ext}}/+\text{ fly.}$ (B) $UAS-Ed^{\text{Ext}}/69B\text{ GAL4 fly wing have irregular wing vein II.}$ (C) $T80\text{ GAL4/+; UAS-Ed}^{\text{Ext}}/+\text{ fly wing exhibits irregular wing vein II and rare notches (indicated by the bracket) in the distal wing margin.}$ (D) Partial dorsal thorax of control ($UAS-Ed^{\text{Ext}}/+\text{) fly showing the normal number of sensory bristles on the scuillum.}$ (E) Scutellums of $T80\text{ GAL4/+; UAS-Ed}^{\text{Ext}}/+\text{flies show extra sensory bristles (indicated by arrowheads).}$ The extra sensory bristle phenotype is more evident when $pnr\text{ GAL4}$ is used to mediate $Ed^{\text{Ext}}$ expression. A loss of epithelium is also noticeable (F). (G) shows a magnification of a part of the mesothoracic region of the thorax in (F). The microchaetae are missing (asterisk) or quadrupled (arrows).
Figure 3. $4 \text{Ed}^{\text{Ext}}$ has dominant-negative activity
Figure 3. 5: Genetic interactions between ed and genes of the N signaling pathway. Anti-HRP labeling of neurons in the embryos (A-H). (A-D) show lateral views and (E-H) show ventral views. Embryos in (A-H) were grown at 29°C. (A and E) Wild type embryos. (B and F) Examples of the extent of hyperplasia of the CNS evident in ed^{2B8}/ed^{ts} embryos. Ectopic expression of N^{act} in the parasegments 4-6 mediated by the Kr-GAL4 driver, results in the suppression of neuronal cell fate in this region (C and G). ed^{ts}:UAS-N^{act}/ed^{2B8}; Kr-GAL4/+ embryos show strong suppression of the N^{act} overexpression phenotype resulting in a near wild type morphology (D and H). (I) Control wing of Dl^{via1}/+ fly. (J) ed^{ts}/ed^{m1} lies grown at 25°C show a mild thickening of wing vein II. (K) ed^{ts}/ed^{m1}; Dl^{via1}/+ flies grown at 25°C show an enhancement of the thick wing vein II phenotype and show additional wing vein material (indicated by the arrowhead) in the posterior cell. (L) ed^{ts}/+ heterozygous flies raised at 29°C show a wild-type wing morphology. (M) E(spl)^{8D06}/+ flies raised at 29°C show extra wing vein material (arrowhead) in the posterior cell with low penetrance. (N) ed^{ts}/+; E(spl)^{8D06}/+ flies at 29°C display more ectopic vein material (arrowhead) with full penetrance.
Figure 3. 5: Genetic interactions between *ed* and genes of the Notch signaling pathway.
Figure 3. 6: The dominant negative activity of $Ed^{Ext}$ is modulated by the N signaling pathway. (A-D) SEM of anterior midline region of adult thorax. (A) Normal microchaetae distribution in wild type flies. Ectopic expression of EdExt mediated by $Eq\text{-}GAL4$ driver results in a mild increase in the microchaetae density (B). Ectopic expression of Dl also results in an increased microchaetae density (C). (D) $UAS\cdot Ed^{Ext}/+; UAS-Dl/Eq\text{-}GAL4$ flies show significantly increased microchaetae density. (E) Arrangement of sensory bristles on the notum of an $UAS\cdot Ed^{Ext}/+$ fly. An extra bristle can be seen rarely. $pnr\text{-}GAL4$ mediated ectopic expression of $Ed^{Ext}$ results in formation of extra bristles which is accompanied by loss of epidermis (F). Overexpression of E(spl)m7 results in suppression of sensory bristle formation, resulting in a bald mesothorax region (G). Overexpression of E(spl)m7 completely suppresses the phenotype of $Ed^{Ext}$ overexpression (H). Thus $UAS\cdot Ed^{Ext}/+; UAS-E(spl)m7/pnr\text{-}GAL4$ flies (H) are indistinguishable from $UAS-E(spl)m7/pnr\text{-}GAL4$ flies (G).
Figure 3. 6: The dominant negative activity of Ed\textsuperscript{Ext} is modulated by the Notch signaling pathway.
CHAPTER 4

*friend-of-echinoid* limits neurogenesis in the wing disc and interacts with the Notch signaling pathway

**INTRODUCTION**

Echinoid (Ed), a transmembrane IgC2 type cell adhesion molecule has been shown to be involved in negatively regulating the EGFR pathway during eye morphogenesis. *ed* loss of function mutations result in extra photoreceptor formation (Bai et al., 2001; Rawlins et al., 2003b; Spencer and Cagan, 2003). *ed* is required to suppress neural specification during embryonic neurogenesis and to inhibit SOP formation. Ed has been shown to synergize with the Notch signaling pathway in these processes (Ahmed et al., 2003). Analysis of the *Drosophila* genomic sequence revealed the presence of a gene with a high degree of similarity to *ed* called *friend-of-echinoid* (*fred*) (Flybase, FBrf0146436; R. Cagan, personal communication). *fred* is present adjacent to *ed* on the second chromosome which implies that these two genes might be the result of a duplication of an ancestral gene. Although the loss of *ed* results in an embryonic neural hyperplasia phenotype, it is substantially weaker than the phenotype observed for other neurogenic mutants. It is possible that this comparatively mild phenotype is due to the presence of wild type *ed* from maternal deposition. However, it is also possible that *fred* has a role in
suppressing neurogenesis and thus can functionally compensate for loss of ed resulting in a weaker phenotype. Consistent with this possibility, embryos transheterozygous for ed^{2B8} and a deficiency, Df(2L)edsz, that is missing both ed and fred genes show a much stronger phenotype than ed^{2B8}/ed^{2B8} embryos. fred cDNA clones available from the BDGP demonstrate that fred is transcribed. In situ RNA hybridization for fred transcript shows that fred is expressed ubiquitously in embryos and wing discs. In order to decipher whether fred has a function during neurogenesis, I generated fred RNAi transgenic lines. Here I report on the role of fred in the process of SOP specification. Our results show that fred gene function is required for normal development of sensory organs, the adult epidermis and the eye. In these processes, fred seems to be closely interacting with the Notch signaling pathway as well as with its parologue ed.

RESULTS

fred is a paralogue of echinoid

Fly BLAST revealed a novel gene, fred, with high sequence similarity to ed. fred is located proximal to ed, which maps at 24D3-4 on chromosome arm 2L, and is arranged in the opposite orientation to ed (Figure 4.1 A).

Conceptual translation of the largest open reading frame (ORF) of the fred cDNA (ext.CG3390) (see Materials and Methods), predicts a protein of 1198 amino acids (Figure 4.1C). This protein product has a putative signal sequence, seven immunoglobulin (Ig) C2 type domains (Williams and Barclay, 1988), followed by two fibronectin type III (Fn type-III) domains (Hynes, 1986), a transmembrane domain, and a 188 amino acid C-terminal region with no readily identifiable structural or functional
motif. Immunoglobulin domain containing proteins are frequently involved in cell-cell adhesion and signal transduction.

Examination of Ed sequence indicates the presence of an additional Ig C2 domain upstream to the first IgC2 domain and another Fn type-III domain downstream to the Fn type III domain (data not shown). *fred* appears to be a paralogue of *ed* as the overall structural arrangement of Fred closely mimicks that of Ed. Both proteins contain seven Ig C2 type domains, two Fn type-III domains followed by a transmembrane domain and an intracellular region. The Ig C2 domains of Fred exhibit a high sequence similarity to the corresponding Ig C2 domains of Ed, ranging from 62 to 91 percent identity (Figure 4. 2A). In contrast, Ig C2 domain similarity between unrelated Ig C2 proteins are generally in the 40 percent range. The proteins also exhibit sequence similarities in regions between the Ig C2 domains. The overall identity between the extracellular regions of the two proteins is 69 percent. In contrast, the putative intracellular regions of the two proteins exhibit only limited sequence similarity (30 percent identity).

BLAST searches of the recently completed Mosquito, *Anopheles gambiae*, genome sequence (Holt et al., 2002) using Ed and Fred, revealed the presence of two highly similar genes. The similarity is particularly evident in the comparison of individual IgC2 domains (Figure 4. 2A). Like *ed* and *fred* these genes are arranged in tandem, but the predicted transcription units are in the same orientation. Thus, while the predicted Mosquito *ed/fred* genes appear to be transcribed from the same strand, the *Drosophila ed* and *fred* transcription units are transcribed in opposite directions (Figure 1A). Direct comparison of the predicted amino acid sequences of *Drosophila* Fred and Ed with the *Anopheles* orthologs shows a significantly higher overall similarity of both
predicted *Anopheles* proteins to *Drosophila* Ed. This suggests that *Drosophila* Ed is likely to be closer in sequence to the ancestral gene than Fred. This possibility is further supported by the observation, that both predicted *Mosquito* Ed/Fred proteins show sequence similarity to the entire Ed intracellular region, but only limited similarity to the Fred intracellular domain (Figure 4.2B).

**fred mRNA expression**

The *fred* mRNA expression pattern was determined by *in situ* hybridization. *fred* shows a rather general expression pattern. In the embryo, *fred* is expressed in most tissues, including the central nervous system (CNS) and epidermis (Figure 4.3A-C). In third instar larval wing and eye discs *fred* is also rather uniformly expressed (Figure 4.3D and F). Control staining with sense probe does not show a detectable signal (Figure 4.3E).

**fred function is required for proper adult sensory organ and epidermal development**

In order to study the function of *fred*, we have used the heritable and inducible double stranded RNA-mediated interference (RNAi) method (Tavernarakis et al., 2000). For this study, transcript sequence of *fred* was cloned as a dyad symmetric molecule in the pUAST vector and transgenic lines established. Expression of the construct was induced by crossing the transgenic lines to a tissue and/or stage specific *GAL4* driver lines (Kennerdell and Carthew, 2000). Transcription of a dyad symmetric molecule results in a RNA that snaps back to give rise to a dsRNA with a hairpin loop, which mediates the degradation of the corresponding endogenous mRNA (Sijen et al., 2001).
638 bp region of \textit{fred} was selected for this analysis based on minimal similarity to \textit{ed} sequence (see Materials & Methods).

The effectiveness of the \textit{UAS-fred RNAi} construct in mediating the degradation of \textit{fred} transcripts was tested in third-instar larval wing discs using the \textit{pannier-GAL4 (pnr-GAL4)} driver (Figure 4. 4C; Heitzler 1996b). \textit{pnr-GAL4} mediated expression of the \textit{UAS-fred RNAi} construct in the dorsal most region of the wing disc results in a strong reduction of \textit{fred} mRNA (Figure 4. 4B) as compared to \textit{pnr-GAL4/ +} control animals (Figure 4. 4A). Staining for \textit{ed} mRNA or protein did not show any decrease in expression (data not shown), verifying the specificity of the \textit{fred RNAi} construct. Many of the \textit{pnr-GAL4/ UAS-fred RNAi} larvae develop into adults that display a range of phenotypes including, a loss of epithelium resulting in a smaller notum and scutellum, and loss or duplication of sensory bristles (Figure 4. 5 B and D). These phenotypes are generally more severe when these flies are raised at 29°C. Here, loss of epithelium is so extensive that approximately one-third of the eclosed adults have holes in the dorsal cuticle (Figure 4. 5 E). In addition, a third of the pharate adults fail to eclose and display defects in dorsal cuticle (not shown). A similar phenotype is also observed using the \textit{Eq-GAL4} driver, which directs expression in the anterior region of the future notum, with a stronger expression in the anterior midline (Pi et al., 2001). Degradation of \textit{fred} mRNA in this region also results in the loss of epithelial tissue, resulting in a pinched appearance of the notum (Figure 4. 5G).

The loss of epithelia and the misspecification of sensory bristles might indicate a role of \textit{fred} in sensory organ formation and/or in cell survival. To test these possibilities, we followed sensory organ formation by analyzing the expression of the SOP markers
neur-A101lacZ and SRV-lacZ. A101 is an early marker for the SOP cell fate; in wild type wing discs, it labels a single nucleus in each proneural cluster (Huang et al., 1991) (Figure 4. 6C and E). SRV-lacZ is a sc lacZ reporter construct that specifically labels the SOPs (Figure 4. 6G). Suppression of fred function in the dorsal most part of the wing discs results in a dramatic increase in the number of cells expressing the A101 SOP marker (Figure 4. 6F). The ectopic A101 positive cells are generally arranged in a single large, continuous patch. Ectopic expression of the SOP marker A101 was also observed when another GAL4 driver, apterous-GAL4 (ap-GAL4), which drives expression in almost the entire dorsal compartment of the wing disc (Figure 4. 6A), was used (Figure 4. 6D). Ectopic SOPs were also obtained in the UAS-fred RNAi; pnr-GAL4/SRV-lacZ wing discs (Figure 4. 6H). We tested whether the proneural genes ac and sc are required for the specification of these ectopic SOPs by using the Df(1)sc10-1 line (Lindsley and Zimm, 1992). Males hemizygous for this deficiency lack both ac and sc and have a bald nota as no SOPs are specified. The induction of ectopic SOPs upon fred suppression require ac and sc as male pharate adults and the occasionally eclosed adults of the genotype Df(1)sc10-1/Y; UAS-fred RNAi/pnr-GAL4 show no bristles and have a near wild type notum morphology (Figure 4. 6I). The specification of ectopic SOPs upon fred suppression is accompanied by extensive cell death, as revealed by acridine orange staining (Figure 4. 6J and K), likely amounting to loss of sensory structures and epithelial tissue in the adult fly.

We also analyzed the effects of fred RNAi in the developing eye. At 25°C, flies transheterozygous for UAS- fred RNAi and the eye-antennal disc specific driver line
GMR-GAL4 show fused ommatidia and mispositioned and/or missing bristles. Again this phenotype is enhanced if flies are raised at 29°C (Figure 4. 6I).

**fred genetically interacts with the Notch signaling pathway**

The Notch signaling pathway is involved in limiting the SOP fate to a single cell within each proneural cluster (Artavanis-Tsakonas et al., 1999; Bray, 1998). Since degradation of *fred* mRNA leads to formation of ectopic SOPs, we wanted to see if the Notch signaling pathway genes functionally interact with *fred* in this process and, thus, may modulate the *fred* RNAi phenotype. To this end, we tested four Notch pathway genes, *Notch* (*N*), *Suppressor of Hairless* (*Su(H)*), *Hairless* (*H*) and *E (spl) m7* for genetic interactions with *fred*.

Over-expression of Notch leads to a loss of sensory organs and hair to socket transformation (Frise et al., 1996; Guo et al., 1996; Lieber et al., 1993). Expression of a *UAS-Notch (UAS-N)* construct with *pnr-GAL4* results in flies that show loss of most of the bristles from the dorsal most region of the thorax (Figure 4. 7C). In addition, occasionally bristle to socket transformation is observed. When *fred* dsRNA and Notch were expressed simultaneously using the *pnr-GAL4* driver, the flies showed a phenotype that is intermediate between that of the two individual phenotypes (Figure 4. 7D). Although over-expression of Notch could suppress the cuticular holes and ectopic microchaeta formation, the thoraces of these flies still had some of the phenotypes associated with RNAi mediated suppression of *fred*, such as a pinched notum and a smaller scutellum.
Following Notch activation, the $\text{N}^{\text{icd}}$ translocates to the nucleus where it forms a complex with the transcription factor $\text{Su}(\text{H})$ and switches on the transcription of $E$ ($spl$) complex (Artavanis-Tsakonas et al., 1999). Loss of $\text{Su} \ (\text{H})$ results in the formation of ectopic sensory bristles, while over-expression results in suppression of sensory organ specification (Schweisguth and Posakony, 1994). Ectopic expression of $\text{Su}(\text{H})$, using the $\text{pnr-GAL4}$ driver, results in the absence of sensory organs in the medial region of the notum (Figure 4. 7E). Simultaneous expression of both $\text{Su}(\text{H})$ and the $\text{fred}$ RNAi construct in the $\text{pn}r$ domain produces flies that are similar to the $\text{UAS-Su}(\text{H}); \text{pnr-GAL4}$ flies (Figure 4. 7F). Moreover, the ectopic cell death associated with $\text{fred}$ suppression was alleviated by $\text{Su}(\text{H})$ overexpression (Figure 4. 7I-K). Thus, ectopic expression of $\text{Su}(\text{H})$ effectively suppresses the phenotype associated with the reduction of $\text{fred}$ function. We also tested the effect of loss of $\text{Su}(\text{H})$ function on the $\text{fred}$ RNAi phenotype. Reduction of one functional copy of $\text{Su} \ (\text{H})$ in $\text{UAS-fredRNAi/pnr-GAL4}$ flies did not show a consistent modulation of the phenotype, indicating that this assay might not be sensitive enough. However, eye morphogenesis has been proven to be very sensitive to dosage sensitive interactions (Bai et al., 2001; Carthew et al., 1994; Therrien et al., 2000). Therefore, we determined the effect of loss of one functional $\text{Su} \ (\text{H})$ copy on the rough eye phenotype generated by expression of $\text{UAS-fred RNAi}$ in eye with the $\text{GMR-GAL4}$ driver. A consistent enhancement of the $\text{fred}$ RNAi induced rough eye phenotype was observed upon decreasing $\text{Su} \ (\text{H})$ function (Figure 4. 8C).

Hairless ($\text{H}$) is a negative regulator of the Notch pathway (Bang et al., 1995). $\text{H}$ antagonizes Notch target gene activation by binding to the Notch signal transducer, $\text{Su}(\text{H})$ (Schweisguth and Lecourtois, 1998). Accordingly, over-expression of $\text{H}$
phenocopies reduction of Notch activity (Bang et al., 1995; Lyman and Yedvobnick, 1995). Ectopic expression of H in the \( pnr \) domain results in the formation of multiple/split bristles and loss of epidermal tissue. This phenotype is enhanced in animals with suppressed \( fred \) activity in the \( pnr \) domain (data not shown). Functional interactions between \( H \) and \( fred \) are also evident in the eye. \( UAS-H/GMR-GAL4 \) flies have eyes that are slightly smaller along the anterior-posterior axis and show ommatidial fusion and interommatidial bristle tufting, as well as bristle loss (Figure 4.8E). When \( fred \) activity is suppressed in this genetic background, there is an enhanced disruption of the eye morphology. Ommatidia lack definition, bristle tufting is more severe and loss of bristles is observed (Figure 4.8F).

Among the best characterized targets of Notch signaling in \( Drosophila \) are the seven \textit{Enhancer of split} \( \{E (spl)\} \) complex genes (Klambt et al., 1989; Knust et al., 1992). Activation of the \textit{Notch} signaling pathway results in the activation of the expression of various \( E(spl) \) complex genes (Bailey and Posakony, 1995; Jennings et al., 1994; Lecourtois and Schweisguth, 1995). Over-expression of \( E (spl)m8, E (spl)m7, E (spl)m\beta, E (spl)m\gamma, E (spl)m 3 \) and \( E (spl)m\delta \) in wing discs results in loss of sensory organs (Ligoxygakis et al., 1999). To determine whether the phenotype associated with suppression of \( fred \) could be modulated by expression of an \( E(spl) \) complex gene, we expressed \( E (spl) m7 \) simultaneously with \( fred \) dsRNA using the \( pnr-GAL4 \) driver (Figure 4.8G). Flies that over-express both \( fred \) dsRNA and \( E (spl) m7 \) were indistinguishable from those expressing only \( E (spl) m7 \) (Figure 4.8H). Third instar larval wing discs from these crosses were also analyzed for \( A101-lacZ \) expression. Ectopic expression of \( E(spl)m7 \) by \( pnr-GAL4 \) results in the loss of dorsocentral and scutellar SOPs, while
suppression of *fred* activity results in large domains of A101 positive cells. Notably, wing discs of *UAS-E(spl)m7; UAS-fred RNAi/pnr-GAL4: A101-lacZ* larvae showed the same SOP pattern as *UAS-E(spl)m7; pnr-GAL4: A101-lacZ* larvae (data not shown). So ectopic expression of *E(spl)m7* suppresses the phenotype associated with the reduction of *fred* in the wing disc.

We tested whether this is also the case in the developing eye. Degradation of *fred* mRNA in the eye with *GMR-GAL4* results in a rough eye phenotype with missing or duplicated bristles and fused ommatidia (Figure 4. 8G). Ectopic expression of *E(spl)m7* by *GMR-GAL4* results in the loss of most of the bristles in the eye. While the ommatidia remain highly organized, bristle sockets are present only infrequently or are entirely missing. If present, sockets are mispositioned and sometimes duplicated (Figure 4. 8H). The phenotype of eyes of animals expressing both, *E(spl)m7* and *fred* ds RNA under the control of *GMR-GAL4*, is very similar to the phenotype of *UAS-E(spl)m7/GMR-GAL4* flies, with the exception of a few fused ommatidia that can still be observed in the posterior part of the eye (Figure 4. 8I).

**Genetic interaction between *fred* and *ed***

*fred* shares high sequence similarity with *ed*. *fred* and *ed* are both uniformly expressed in third-instar larval eye and wing discs. To address the possibility that *ed* and *fred* are functioning in close concert we employed a dosage sensitive genetic interaction assay. *ed^{2B8}* is an amorphic allele of *ed*. Flies carrying only one functional copy of *ed* and one copy of the *GMR-GAL4* driver(*ed^{2B8}/GMR-GAL4*) show near wild-type morphology (Figure 4. 9A). RNAi mediated suppression of *fred* in the developing eye results in a mild
rough eye phenotype (Figure 4. 9B). In contrast, suppression of \textit{fred} in eye-antennal discs of animals with only one functional allele of \textit{ed} (\textit{ed}^{2B8}/\textit{GMR-GAL4};\textit{UAS-fred RNAi}/+) leads to a severe rough eye phenotype (Figure 4. 9C), which is easily distinguishable from that of \textit{GMR-GAL4}; \textit{UAS-fred RNAi} flies (Figure 4. 9B). The ommatidial fusion seen in \textit{GMR-GAL4}; \textit{UAS-fred RNAi} eyes is significantly enhanced, and there is increased bristle loss as well as pitting and scarring of the ommatidia of \textit{ed}^{2B8}/\textit{GMR-GAL4}; \textit{UAS-fred RNAi} flies.

Since \textit{ed} has been shown to be a negative regulator of the EGFR pathway, we also tested for dosage sensitive interaction with members of this pathway. \textit{Gap1} (GTPase-activating protein) is a negative regulator of the EGFR pathway. EGFR signaling is transduced by the Ras/Raf/MAP kinase cascade. \textit{Gap1} inactivates \textit{RAS1} (\textit{RAS1} is activated by exchanging GDP for GTP) by stimulating its intrinsic GTPase activity (Gaul et al., 1992). Reduction of \textit{Gap1} in the \textit{GMR-GAL4} background does not result in any eye abnormality (data not shown). Reduction of \textit{Gap1} in \textit{UAS-fred RNAi}/+; \textit{GMR-GAL4}/+ flies resulted in a moderate enhancement of the rough eye phenotype seen in \textit{UAS-fred RNAi}/+; \textit{GMR-GAL4}/+ flies (Figure 4. 9, compare E to D). This enhancement, however, was not very consistent as only 30 percent of \textit{UAS-fred RNAi}/+; \textit{GMR-GAL4}/+; \textit{Gap1}\textit{B2}/+ flies showed increased roughness (Figure 4. 9E) and the rest 70 percent showed no significant change (Figure 4. 9F). \textit{Pointed} is a downstream effector of EGFR signaling pathway (Brunner et al., 1994). \textit{GMR-GAL4} /+ \textit{pnt}^{A88}/+ flies have a normal eye morphology (data not shown). \textit{UAS-fred RNAi}/+; \textit{GMR-GAL4} /+; \textit{pnt}^{A88}/+ flies show a suppression of the rough eye phenotype caused by \textit{fred RNAi}. Again, this suppression was not very consistent as only 40 percent of the \textit{UAS-fred RNAi}/+; \textit{GMR-GAL4}/+; \textit{pnt}^{A}
+/flies showed this suppression (Figure 4. 9G). While genetic interactions were observed between fred and the two members of the EGFR pathway, these interactions were consistently weaker than that observed with the Notch signaling pathway. We also monitored EGFR activity by staining for the doubly phosphorylated mitogen-activated protein (MAP) kinase (dp-ERK; Gabay,1997). While we could consistently detect the wild type expression pattern of dp-ERK in the wing discs, we did not observe a significant change in the dp-ERK expression in the wing discs of either UAS-fred RNAi; pnr-GAL4 or UAS-fred RNAi; ap-GAL4 larvae (data not shown).

**DISCUSSION**

*fred is a paralogue of ed*

The predicted fred and ed proteins are highly similar. The protein products of both genes contain a putative signal sequence, seven highly conserved Ig C2 domains, followed by two Fn-type III domains. The strong conservation observed for the entire extracellular regions of Fred and Ed is contrasted by a rather limited similarity between the respective intracellular regions. Thus, the putative intracellular domains of the two proteins are approximately 30 percent identical over a length of 188 amino acids, whereas the extracellular region is 69 percent identical.

The *fred* and *ed* transcription units are located, in reverse orientation, approximately 100 kilobases apart on chromosome arm 2L. The close proximity and sequence similarity of the two genes suggests that a duplication of the original gene has occurred. The presence of two putative transcription units in the *Anopheles* genome, that encode highly similar proteins, indicates that such a duplication would have happened
before the evolutionary separation of the ancestors of *Anopheles* and *Drosophila*. Comparison of the *Drosophila* and *Anopheles* genes furthermore shows, that in *Anopheles* the two transcription units are arranged head to tail, i.e. the two genes appear to be transcribed from the same strand, while in *Drosophila* a head to head arrangement is observed. This suggests that, after the lineages leading to present day *Drosophila* and *Anopheles* split, an inversion event has taken place. Both tandemly repeated transcription units in *Anopheles* encode proteins with significantly higher similarity to *Drosophila* Ed than to *Drosophila* Fred (Figure 4.2A and B). The observation of a co-linear sequence similarity of *Anopheles* Ed/Fred with *Drosophila* Ed, but not Fred, suggests that Ed is closer in structure to the original duplicated gene. It is, furthermore, tempting to speculate whether the absence of the C-terminal region in *Drosophila* Fred could represent a consequence of an inversion event that did not include the entire ancient *fred* gene. The sequence similarity of both *Anopheles* Ed/Fred genes is higher to *Drosophila* Ed than to *Drosophila* Fred (Figure 4.2A). This is particularly pronounced in the putative intracellular domains of these proteins (Figure 4.2B). The divergent structure of the putative *Drosophila* Fred intracellular domain also raises the question, whether or not the *Drosophila* Fred has undergone a functional divergent evolution compared to its *Anopheles* counterpart(s) and *Drosophila* Ed. If the diversity in the intracellular region of *Drosophila* Fred indeed represents a functional specialization, however, remains to be determined.
**Fred function is required for proper development of adult sensory structures**

Using inducible RNAi, we have shown that Fred function is required in eye morphogenesis and to restrict SOP cell fate in wing disc. Suppression of Fred function in the developing wing disc results in ectopic SOPs, as revealed by the SOP markers, neur-A101-lacZ and SRV-lacZ. In the wing discs of the mid-late-third-instar larva, only few SOPs are present (Figure 4. 6C). However, ap-GAL4 driven degradation of Fred mRNA results in specification of a continuous patch of A101 lacZ expressing cells in the wing pouch region (Figure 4. 6D). In the case of pnr-GAL4 driven Fred mRNA degradation, SOPs are induced at positions where in the wild type wing disc, no SOPs exist yet (Figure 4. 6E and F). Similar results are obtained with SRV-lacZ, a SOP marker (Figure 4. 6G and H). However, normally, SOPs do form in these regions of the wing disc at later stages of development. Thus, suppression of Fred function may result in precocious formation of SOPs. Moreover, the presence of the ectopic SOPs in large, continuous patches, without any intervening epidermal cells, indicates a disruption of the process of lateral inhibition. Adult flies of the UAS-fred RNAi/+; pnr-GAL4: A101neur-LacZ/+ genotype show a moderate increase in the number of microchaeta (Figure 4. 5D). These extra microchaeta could originate from the ectopic SOPs. Furthermore, frequent bristle duplications are also observed. These phenotypes suggest that Fred function might be required during SOP specification and bristle development.

In our experiments, specific regions of the wing disc appeared to be more sensitive to suppression of Fred function, as indicated by the positions occupied by ectopic SOPs. While pnr-GAL4 drives expression in the dorsal most region of the wing disc, only some regions in the pnr domain of wing discs of UAS-fred RNAi/+; pnr-GAL4:
A101neur-LacZ/+ larvae show ectopic expression of the SOP markers, A101 and SRV-lacZ (Figure 4. 6F and H). The same observation was made using ap-GAL4. ap-GAL4 drives expression of UAS constructs in almost the entire dorsal domain, however, in ap-GAL4/+;UAS-fred RNAi: A101neur-LacZ/+ third-instar-larvae, ectopic expression of the SOP marker A101-lacZ is only detected in a part of that region (see Figure 4. 6A and D). These observations might point to a higher requirement for fred function in certain regions of the wing disc, and/or slightly different levels in the expression by the respective GAL4 drivers that would result in different levels of fred mRNA degradation.

RNAi mediated suppression of fred also results in an increase in cell death. Presently, it is not clear whether this is a direct or indirect consequence of cell fate changes associated with the formation of ectopic SOPs, which subsequently undergo cell death, or if there is a separate requirement for fred function in epidermal cells. However, the strong suppression of cell death upon overexpression of Su(H) and the wild type morphology of the notum of males lacking ac and sc, strongly suggest that the ectopic cell death is associated with the change in cell fate.

Fred interacts with the Notch signaling pathway

The observations that changes in the activity of four genes of the Notch signaling pathway can either suppress or enhance the phenotypes associated with the suppression of fred function suggest that fred is functioning in close concert with the Notch signaling pathway. Reduction in the activity of a Notch signaling pathway gene, Su(H) results in an enhancement of the fred RNAi phenotype. In contrast, ectopic expression of Notch signaling pathway genes, Notch, Su(H) and E(spl)m7 suppresses, to different degrees,
different aspects of the *fred* RNAi phenotype in the developing wing, thorax and eye. Over-expression of Hairless, a negative regulator of the Notch pathway, on the other hand, enhances the phenotypes induced by Fred suppression. It is presently not clear whether Fred defines a separate pathway for SOP determination or if it shares downstream components of the Notch signaling pathway. The remarkable degree to which ectopic expression of an *E(spl)* complex bHLH transcription factor results in a nearly complete suppression of phenotypes associated with *fred* degradation strongly supports the idea of very close functional interactions. These observations, furthermore, raise the possibility that *E(spl)* complex genes and/or other genes of the Notch signaling pathway act downstream of *fred* function.

**fred and ed interact functionally**

Reduction in *ed* gene dosage resulted in a very pronounced, dominant enhancement of the *fred*-RNAi eye phenotype despite the fact that *ed* \textsuperscript{2B8} has no dominant visible phenotype. This suggests that *ed* and *fred* closely interact in processes that require Fred function. The similarity in protein structure and overlapping expression patterns would support such a functional interaction and may also point to the possibility of functional redundancy. Both Ed and Fred contain highly similar Ig C2 domains in their respective extracellular regions. Ig C2 domains are frequently involved in homophilic or heterophilic interactions with other Ig domain containing adhesion molecules (Hortsch, 1996). Thus it is possible that Fred and Ed might communicate via interactions of their extracellular domains. Future research will have to address this possibility.
The weak genetic interaction observed between *fred* and two members of the EGFR pathway also links *fred* to the EGFR pathway, however, analysis of additional components of the EGFR pathway are necessary to determine *fred*'s role in the EGFR signaling.

In summary, suppression of *fred* function results in specification of ectopic SOPs in the wing disc and a rough eye phenotype. Overexpression of N, Su(H) and E(spl)m7 suppresses the *fred* RNAi phenotypes. Accordingly, decreasing Su(H) or overexpression of H enhances the *fred* RNAi phenotypes. Thus *fred*, a parologue of *ed*, is a new gene that shows close genetic interactions with the Notch signaling pathway.
Figure 4. 1: Genomic organization and sequence of *fred*. (A) *fred* is located on chromosome arm 2L at 24D2 appx. 100kb upstream of *ed*. Arrows indicate the direction of transcription. (B) *fred* cDNA clones. Filled boxes represent the *fred* open reading frame (ORF), while the open boxes indicate untranslated regions (UTR). The exon/intron arrangement is indicated. The introns are not drawn to scale. (a) *fred* extendedCG3390 cDNA has an ORF of 3594bp, a 378bp 5'UTR and a 757bp long 3’UTR. Exons I-IX are 407bp, 202bp, 260bp, 198bp, 2232bp, 674bp, 367bp, 348bp and 63bp long. Introns 1-8 are 42,694bp, 16,450bp, 272bp, 9514bp, 107bp, 1684bp, 3392bp and 5401bp long. (b) *fred* SD cDNA clone. 5’UTR is 5324bp long, the longest ORF is 2275bp long and is followed by a 2596bp long 3’UTR. (C) Predicted amino acid sequence of Fred. The seven IgC2 domains and the two Fn III domains are underlined. Transmembrane region (TM) is boxed. The C-terminal region is 188 amino acids long.
Figure 4.2: (A) Alignment of Ig C2 domains of Ed, Fred and Mosquito Ed/Fred 1 and 2. Consensus IgC2 domain amino acids are in bold. Amino acids conserved between all four proteins are boxed.
### Figure 4. 2 continued

#### B

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Figure 4. 2: Alignment of intracellular regions of Ed, Fred and predicted Mosquito Ed/Fred 1 and 2. (B) Amino acids conserved between all four proteins, and between Ed, Ag Ed/Fred 1 and 2 after the asterisk are boxed. Shaded amino acids are conserved between Ed or Fred and at least one of the two Mosquito orthologs.
Figure 4. 3: RNA expression pattern of *fred*. (A-C) *fred* RNA expression in wild type embryos at late stage 12 (A), stage 14 (B) and stage 17 (C). *fred* transcripts are detected in most tissues including the CNS and the epidermis. (D and F) *fred* RNA is ubiquitously expressed in late third instar larval wing (D) and eye discs (F). Control *in situ* hybridization on wild type late third instar larval wing disc using the sense probe shows no expression (E).
Figure 4. 4: RNAi mediated suppression of fred RNA expression in the wing disc. Anterior is to the left and ventral is up. fred RNA expression in a wild type third instar larval wing disc (A) and a UAS-fred RNAi; pnr-GAL4 larval wing disc (B). fred RNA expression is significantly reduced in the dorsal-most part (indicated by the bracket) of the wing disc. (C) X-gal staining of pnr-GAL4/UAS-lacZ wing disc shows the expression domain of the pnr-GAL4 driver.
Figure 4.5: Adult phenotypes associated with \textit{fred} suppression. Anterior is up in A-G. (A) Dorsal thorax of a control fly (\textit{pnr-GAL4/+}). An extra macrochaetae is occasionally present on the scutellum. Small black dots indicate the \textit{pnr} expression domain on the thorax. Big black dots mark the dorsocentral bristles. (B) \textit{pnr-GAL4/UAS-fred RNAi} flies have smaller notum and scutellum and show a loss of dorsocentral macrochaeta. Big black dots indicate the approximate position of the missing macrochaeta. (C and D) are magnified images of the medial thorax of flies in (A) and (B), respectively. \textit{pnr-GAL4/UAS-fred RNAi} flies exhibit extra microchaeta, although the size of individual microchaeta is generally reduced. Duplications of microchaeta (arrowhead) are also evident. (E) \textit{pnr-GAL4/UAS-fred RNAi} flies, raised at \(29^\circ\text{C}\), show a more extensive loss of tissue. The anterior dorsocentral bristles and the microchaeta appear to have disappeared together with the epithelium. Frequently the loss of epidermis results in holes (arrow) in the cuticle covering the dorsal thorax. (F) Anterior midline region of a control fly (\textit{Eq-GAL4/+}). (G) Suppression of \textit{fred} in the presumptive anterior midline region of the larval wing disc in \textit{UAS-fredRNAi/+; Eq-GAL4/+} animals results a pinched appearance, apparently due to loss of epidermis. (H) Control eye of a \textit{GMR-GAL4/+} fly shows a regular, uniform arrangement of the ommatidia and the bristles. (I) \textit{UAS-fred RNAi/+; GMR-GAL4/+} flies have eyes with fused ommatidia and missing, or duplicated bristles. Except for B and D, flies were raised at \(29^\circ\text{C}\).
Figure 4. 5: Adult phenotypes associated with *fred* suppression.
Figure 4. 6: Phenotypes associated with RNAi mediated suppression of *fred* in wing discs (A-H) X-gal staining of *lacZ* reporter gene constructs in third instar wing discs. (A) Mid-late-third-instar larval wing disc expressing *UAS-lacZ* under the control of the *ap-GAL4* driver and a late-late-third-instar larval wing disc expressing *lacZ* under the control of *pnr-gal4* driver (B) shows the domain of expression of the GAL4 driver lines. (C) *neu-lacZ* SOP marker expression at early third instar larval stage in a control (*ap-GAL4/++; neur-A101 lacZ/*+) wing disc. (D) *ap-GAL4/++;UAS-fred RNAi :neur-A101lacZ/*+ wing disc shows ectopic SOP marker expression, indicated by the bar, in part of the dorsal compartment. (E) Wild type pattern of SOPs in control (*pnr-GAL4/ neur-A101 lacZ*) late third instar larva wing disc. (F) Wing disc of a *UAS-fred RNAi/+; pnr-GAL4: neur-A101lacZ/*+ third instar larva. Induction of the ectopic expression of the SOP marker A101 lacZ (indicated by the bar) in the dorsal most region is evident. (G) SOP-specific *lacZ* reporter transgene, *SRV-lacZ*, expression pattern in a wild type larval wing disc. (H) Ectopic SOPs are induced in the dorsal most region of wing discs (indicated by the bar) of *UAS-fred RNAi/+; pnr-GAL4/SRV-lacZ* larva. (I) *Df(1)sc10-1/Y; UAS-fred RNAi/pnr-GAL4* animals lack the proneural genes *ac* and *sc* in addition to RNAi mediated suppression of *fred* function. These flies show no loss of cuticle and no bristles. (J) Acridine orange staining of control *pnr-GAL4/+* wing disc showing the normal extent of cell death occurring during morphogenesis. White bracket indicates the domain of *pnr* expression. (K) *pnr-GAL4/ UAS-fred RNAi* wing discs show a very consistent increase in the number of dead cells in the dorsal most region. (Flies in E and F and J and K were raised at 29°C).
Figure 4.6: Phenotypes associated with RNAi mediated suppression of \textit{fred} in wing discs
Figure 4. 7: Functional interactions of *fred* with Notch signaling pathway genes in the thorax. (A) *pnr-GAL4/+* control fly. White dots mark the *pnr* expression domain on the thorax. (B) Thorax of a *pnr-GAL4/UAS-fred RNAi* fly showing tissue loss. (C) *UAS-N/+; pnr-GAL4/+* fly. Ectopic expression of N results in the loss of all microchaeta and most of the macrochaeta in the *pnr* domain. (D) *UAS-N/+; pnr-GAL4/UAS-fred RNAi* flies display an intermediate phenotype. (E) *UAS-Su(H)/+; pnr-GAL4/+* flies show complete loss of sensory organs in the *pnr* domain and an expansion of the dorsal thorax. (F) In *UAS-Su(H)/+ ;UAS-fred RNAi/pnr-GAL4* flies, the *fred* RNAi phenotype is suppressed. (G) *UAS-E(spl)m7/+; pnr-GAL4/+* have no sensory organs in medial dorsal region. (H) *UAS-E(spl)m7/+;UAS-fred RNAi/ pnr-GAL4* flies are indistinguishable from *UAS-E(spl)m7; pnr-GAL4* flies. (I-K) Acridine orange staining of late third instar larval wing discs of the following genotypes: (I) *UAS-Su(H)/+ ; pnr-GAL4/+*, (J) *pnr-GAL4/UAS-fred RNAi* and (K) *UAS-Su(H)/+ ;UAS-fred RNAi/pnr-GAL4*. Overexpression of Su(H) suppresses the ectopic cell death associated with suppression of *fred* function.
Figure 4. 7: Functional interactions of *fred* with Notch signaling pathway genes in the thorax.
Figure 4. 8: Functional interactions of *fred* with Notch signaling pathway genes during eye development. (A) *Su(H)*^AR9/^GMR-GAL4 flies show a near wild type morphology. (B) *GMR-GAL4/+; UAS-fred RNAi/+* flies display a weak rough eye phenotype. (C) *Su(H)*^AR9/^GMR-GAL4; *UAS-fredRNAi/+* flies show an enhancement of the *fred* RNAi eye phenotype. (D) Eye of a *GMR-GAL4/+; UAS-fred RNAi/+* fly. (E) *UAS-H/ GMR-GAL4* flies show a strong rough eye phenotype. (F) In *UAS-H/GMR-GAL4; UAS-fred RNAi* flies, the eye phenotype is stronger than that caused by overexpression of H alone. (G) Eye of a *GMR-GAL4/+; UAS-fred RNAi/+* fly. (H) *UAS-E(spl)m7/GMR-GAL4* flies show a near wild type morphology except that the interommatidial bristles are missing. (I) *UAS-E(spl)m7/ GMR-GAL4; UAS-fred RNAi*. Overexpression of E(spl)m7 suppresses the *fred* RNAi rough eye phenotype to a large extent. B, D and G illustrate the observed range of *fred* RNAi eye phenotype at 25°C.
Figure 4. 8: Functional interactions of *fred* with Notch signaling pathway genes during eye development.
Figure 4. 9: Genetic interaction of *fred* with *ed* and EGFR pathway genes, *gap* and *pointed*. *ed^{2B8}* is an amorphic allele of *ed* (A) *ed^{2B8}/ GMR-GAL4* eyes appear morphologically normal. (B) Eye of a *GMR-GAL4/+;UAS-fred RNAi/+* fly. (C) *ed^{2B8}/ GMR-GAL4;UAS-fred RNAi/+*: heterozygosity for *ed^{2B8}* in the *fred* RNAi background results in a strong enhancement of the rough eye phenotype caused by *fred* suppression. Flies were shifted from 25°C to 29°C after pupa formation. Flies in D-H were raised at 25°C. (D) Eye of *GMR-GAL4/+;UAS-fred RNAi/+* fly. (E and F) Eyes of *UAS-fredRNAi/+; GMR-GAL4/+; Gap1^{B2}/+* flies. Reduction of the *gap* gene dosage to half enhances the *fred* RNAi phenotype in 30 percent of the flies (E) and the rest show no significant change (F). (G and H) Eyes of *UAS-fred RNAi/+; GMR-GAL4 /+; pnt^{A88}/+* flies. The suppression of *fred* RNAi phenotype is observed in 40 percent of the flies (G), whereas 60 percent show no discernible modification (H).
Figure 4. 9. Genetic interaction of *fred* with *ed* and EGFR pathway genes.
CHAPTER 5

Using microarrays to identify genes misregulated upon fred knock down

INTRODUCTION

Fred RNA is ubiquitously expressed in the wing disc. RNAi mediated knock down of fred results in formation of ectopic sensory organ precursors. This ectopic sensory organ precursor formation and associated adult phenotypes can be suppressed by increasing the activity of Notch signaling pathway. Thus, when Notch or Su(H) are overexpressed simultaneously with the fred knock down construct, ectopic SOPs, dorsal cuticular holes and extramicrochaeta phenotypes associated with fred knock down are suppressed although, the overall size reduction of the notum, seen upon fred RNAi, is still evident. When E(spl) m7 or m8, two of the genes of the E(spl) complex are overexpressed in the fred RNAi background, the fred phenotype is almost completely rescued. Thus, increasing the levels of E(spl) genes can completely compensate for lack of or reduction in Fred activity (Chandra et al., 2003).

An aspect of fred RNAi phenotype sets it apart from the ectopic SOPs phenotype observed with mutations in genes of the Notch signaling pathway. Mutations in genes of the Notch signaling pathway, result in a lack of lateral inhibition. Thus, all the cells of the proneural cluster retain neurogenic potential and go on to become neural precursors or SOPs. These ectopic SOPs are, however, limited to the regions where proneural
clusters are present and do not arise in the apparently non neurogenic regions of the wing disc. The ectopic SOPs seen upon Fred knock down arise in regions of the wing disc which apparently have no neurogenic potential. As discussed in chapter 4, lack of Fred activity also results in a lack of lateral inhibition, such that there are no epidermal cells surrounding the ectopic SOPs. So Fred functions at two levels during development of the adult peripheral nervous system. First, Fred is required to suppress the induction of SOP cell fate in cells that are destined for epidermal cell fate (the non-neurogenic regions where no proneural clusters form). Second, Fred is required for lateral inhibition, such that a SOP will be surrounded by non-SOP cells (epidermal cells, by default). Genetic interactions between \textit{fred} and genes of the Notch signaling pathway suggest that Fred synergizes with this pathway to suppress neurogenesis. However, since the Notch pathway does not have a role in suppressing neurogenesis in the non-neurogenic regions of the wing disc, Fred must be performing functions distinct from that of the Notch pathway in the non neurogenic regions of the wing disc. In order to explore the mechanism by which suppression of \textit{fred} results in the induction of neurogenic potential in normally non neurogenic regions of the wing disc, we adopted an approach of global gene expression analysis. We have used microarray analysis to identify genes that are transcriptionally misregulated upon \textit{fred} knock down.

For performing microarray analysis \textit{fred} was knocked down using the \textit{Apterous-GAL4} driver, which mediates expression in almost the entire dorsal region of the wing disc (Figure 5. 1A; (Calleja et al., 1996)). Microarray analysis was performed in triplicate on RNA extracted from \textit{fred} knock down discs and control wing discs. The microarray data revealed a decrease in expression of some of the genes that are positively regulated
by the Notch pathway. The decrease in the levels of these genes is in line with the observed lack of lateral inhibition in *fred* mutants and genetic interaction of *fred* with the Notch pathway. Apart from Notch pathway regulated genes, this analysis revealed some known genes with a defined role in SOP specification/formation, some known genes with no described roles in SOP formation as well as some predicted genes. We have confirmed the changes in expression of some of the genes, predicted to be misregulated by the microarray analysis, by performing *in situ* hybridization. I further investigated the significance of down regulation of one of the genes. Pannier (Pnr), a GATA-1 transcription factor is required for the expression of the *achaete* and *scute* genes. This analysis has identified an unexpected role of *pnr* in neurogenesis. In the following sections, I will be presenting data that suggests that apart from its known role of promoting neurogenesis in certain regions of the wing disc, *pnr* cooperates with Fred to suppress neurogenesis the non neurogenic regions of the wing disc.

**RESULTS**

*fred* knock down results in transcriptional changes

Expression of a *fred* RNAi construct using the *Apterous-GAL4* (*Ap-GAL4*) driver results in the induction of ectopic SOPs (Chapter 5, Figure 5.1C and D). There is a variation in the number of ectopic SOPs; examples of a weak and a strong phenotype are depicted in Figure 5.1C and D. Identification of transcripts whose expression changes with reduced Fred activity might offer clues to the mechanism of Fred function. To identify such transcripts, RNA expression levels of control (*Ap-GAL4; A101*) and experimental (*Ap-GAL4; A101: UAS-fred RNAi*) wing discs were determined using
oligonucleotide microarrays representing approximately 13,500 known and predicted genes in the *Drosophila* genome (Genechip Drosophila Genome Array 1, Affymetrix). 72 transcripts showed a two-fold or higher increase in expression levels upon *fred* knock down (Table 5.1). Amongst these are 4 transcription factors, 27 misc. enzymes, 5 structural proteins, 2 signal transduction proteins, 8 ligand binding or carrier proteins, 11 proteins with miscellaneous functions and 15 predicted proteins with no known structural or functional motif. 62 transcripts showed a reduction of 1.6 fold or higher (Table 5.2). Contained in this list are 18 transcription factors, 2 signal transduction proteins, 14 misc. enzymes, 2 cell proteins, 7 ligand binding or carrier proteins, 13 proteins with miscellaneous functions and 6 predicted proteins with no known structural or functional motif.

**Validation of microarray data**

To confirm that the changes in transcript expression levels observed by the microarray analysis are indeed a reflection of *in vivo* expression levels, *in situ* hybridization was performed. Eight transcripts were selected from the list of upregulated genes and six from the list of downregulated genes to synthesize digoxigenin labeled RNA probes (Table 5.1 and 5.2). Probes were synthesized to detect transcripts of *tetraspanin 42Ed(CG12846)*, *Bearded, CG3074, CG5164* and *Apterous* did not produce a signal in either the control or experimental wing discs. In case of the gene *Bearded*, a *Bearded-lacZ* reporter line was used instead of *in situ* hybridization to determine its expression level/pattern in *fred* RNAi wing discs (Brd 1.5-lacZ; (Singson et al., 1994)). For the other nine transcripts, *in situ* hybridizations signals were observed and analyzed.
Upregulated genes

CG3734 a predicted serine type peptidase is normally expressed in a limited area in the wing disc. Upon fred knock down, its expression is upregulated. CG10916, a ring finger protein, is ectopically expressed when fred function is reduced. Normally it has a minimal expression in the wing disc. CG12505, Zn finger CCHC type, predicted structural protein is massively misexpressed upon fred knock down although normally it is limited to the notum area of the wing disc (Figure 5. 2A-C and E-G). When engrailed-GAL4 (en-GAL4) that mediates expression only in the posterior region of the wing disc is used to express the fred RNAi construct, the misexpression of the genes is limited to the posterior region of the wing discs (Figure 5. 2I-K). Bearded-lacZ reporter expression was also significantly upregulated and misexpressed in the experimental wing discs (Figure 5. 2D and H).

Downregulated genes

The expression pattern of pannier, E(spl)m β, mirror and homothorax in wing discs has been characterized previously (de Celis et al., 1996; Kehl et al., 1998; Kurant et al., 1998; Pai et al., 1998; Ramain et al., 1993). These transcripts showed the expected expression pattern in the control wing discs (Figure 5. 3A-C). In the experimental wing discs, as predicted by the microarray data, there was a significant reduction in their expression levels (Figure 5. 3D-F). Thus, transcripts identified to be misregulated upon fred knock down using microarray analysis were confirmed by in situ expression analysis.
Analyzing the contribution of Pnr to ectopic SOP phenotype induced upon *fred* knock down

Amongst the genes misregulated by *fred* knock down in the wing discs, *pnr* is especially interesting. Pnr, a GATA-transcription factor, is required to upregulate the expression of *ac/sc* in the dorsocentral (DC) and scutellar (SC) proneural clusters. Decreased *pnr* function results in loss of the *ac/sc* expression in the proneural clusters and the corresponding DC and SC SOPs are missing. Overexpression of Pnr leads to extra SOPs to form at the DC and SC positions. Since Pnr is required for the formation of SOPs, we wondered how the decrease in the levels of *pnr* in *fred* RNAi discs affected the ectopic SOP formation.

To study the effect of reduced *pnr* on the *fred* RNAi phenotype, we used a null allele of *pnr*, *pnr*<sup>VX6</sup>, in combination with a hypomorphic allele of *pnr*, *pnr*-GAL4. To mediate *fred* knock down we have used *pnr*-GAL4 driver. Early and late third instar larvae of the genotype *pnr*<sup>VX6</sup>/ *pnr*-GAL4: A101 are shown in Figure 5. 4C and G. As expected, the DC and SC SOPs are missing (Compare to control Figure 5. 4A and E). Early and late third instar larvae of the genotype *UAS-fredRNAi; pnr*-GAL4: A101 are shown in Figure 5. 4B and F. Ectopic patch of A101 expressing cells can be seen. When *fred* knock down is induced using the *pnr*-GAL4 driver in the *pnr*<sup>VX6</sup> background (larvae of the genotype, *UAS-fredRNAi; pnr*<sup>VX6</sup>/ *pnr*-GAL4: A101), a dramatic enhancement of the ectopic SOPs phenotype is observed (Figure 5. 4D). In approximately 62% (n=26) of early third instar wing discs that have very low levels of *pnr* and *fred*, two circular patches of tissue, expressing the A101 SOP maker are observed. In the late third instar larvae of the *UAS-fredRNAi; pnr*<sup>VX6</sup>/ *pnr*-GAL4: A101 genotype (Figure 5. 4H), although, the SOP
phenotype is much stronger than that observed in UAS-fredRNAi; pnr-GAL4: A101 larvae, it is not as dramatic as the phenotype seen in the early third instar larvae. We suspect that the larvae that exhibit this strong enhancement do not survive to late third instar stage and the larvae developing to the late stages are the ones that have a weaker phenotype. The enhancement of the ectopic SOP phenotype seen with the simultaneous reduction of pnr and fred suggests pannier has a role in suppressing SOP formation.

**DISCUSSION**

*fred* knock down in the wing discs results in changes in the transcript levels of a number of genes. For the genes activated by N signaling, changes in their expression levels were expected based on the observed phenotype and genetic interaction with the N pathway. Some of the misregulated transcripts are genes known to be involved in the formation of proneural clusters. Amongst these genes, we analyzed the role of Pnr further and uncovered a novel role of this prepatterning gene.

**Transcripts expressed in response to Notch signaling are downregulated upon *fred* knock down**

In the list of downregulated genes (Table 5.2), the genes of the *E(spl)* complex (*E(spl)-C*) and *vestigial* (vg) are significant as they show that our previous observation of synergy between Fred and the Notch pathway has a transcriptional basis. Genes of the *E(spl)-C* and vg are expressed when Notch signaling pathway is activated (Bailey and Posakony, 1995; Jennings et al., 1994; Kim et al., 1996; Lecourtois and Schweisguth, 1995). There are seven bHLH genes in the *E(spl)-C*: m3, m5, m7, m8, mβ, mδ and
Out of these genes, m3, m7, mβ and mδ are reduced in expression upon fred knock down (Table 5.2 and Figure 5.3). Within the E(spl)-C also occur non-bHLH genes like E(spl) m2, m4 and mα which are predicted to encode proteins with amphipathic α–helical domain. E(spl) m2, m4 and mα are also expressed in response to N signaling (Lai et al., 2000b; Nagel et al., 2000; Wurmbach et al., 1999). Amongst these genes m2 and mα are also reduced in expression levels in fred RNAi discs (Table 5.2).

Microarray analysis of fred RNAi wing discs is yielding expected results. Thus, the genes that might be expected to be down regulated based on the genetic interaction observed between Fred and the N signaling pathway are showing the expected decreased expression. If N signaling requires Fred activity or if the transcriptional output of both N pathway and Fred are the same, then upon fred knock down, N signaling will be reduced or its target gene expression will be reduced resulting in a decreased transcriptional read out of the pathway.

**Genes of/associated with Notch signaling pathway are misregulated**

Big brain is a neurogenic gene (Hartenstein, 1986; Lehmann et al., 1983). It’s loss of function results in an increase in SOPs. It acts cell autonomously to inhibit neurogenesis and is proposed to be on the signal receiving side. Based on overexpression studies, Bib is proposed to act in concert with the Dl and N (Doherty et al., 1997). It is not known how bib expression is regulated. The observed decrease in bib RNA is in line with the observed lack of lateral inhibition in fred mutants.
Bearded (Brd) is one of the six related genes of the Bearded complex (Brd-C). Loss of Brd has no phenotype probably due to its redundant function with the other genes of the Brd-C (Lai et al., 2000a; Lai et al., 2000b). However, Brd gain-of-function allele (Brd\textsuperscript{GOF}) or Brd overexpression results in extra sensory bristles. Brd antagonizes the N signaling pathway via an unknown mechanism. Brd\textsuperscript{GOF} phenotype can be partially suppressed by an extra dose of N (Leviten and Posakony, 1996). To examine Brd expression, a Brd-lacZ reporter was used (Brd 1.5-lacZ; (Singson et al., 1994)). Knock down of fred results in ectopic expression expression of Brd (Figure 5. 2H compare to D). Overexpression of Brd results in formation of extra SOPs due to interference with N activity. Thus, fred knock down might be resulting in decrease in lateral inhibition due to increased expression of N antagonist Brd. Brd has been shown to be upregulated by the proneural proteins Ac and Sc (Singson et al., 1994).

**Genes with known roles in SOP formation are misregulated in fred RNAi discs**

Pannier and genes of the Iroquois complex (Iro-C: araucan, caupolican and mirror) are classified as selector and patterning genes. They are required for the formation of the mesothorax and the lateral thorax, respectively, of the adult fly and are required to prefigure the pattern of SOPs in these regions via positive regulation of ac/sc in the proneural clusters. Decreased function of pnr and genes of the Iro-C results in loss of ac/sc expression from the proneural clusters and absence of the corresponding sensory organs. Pnr is positively regulated by Dpp. Dpp represses Iro-C expression and vein mediated EGFR signaling initiates Iro-C expression (Reviewed in (Gomez-Skarmeta et al., 2003)). Pnr, Araucan and Mirror expression is decreased in the absence of fred (Table...
5. 2; Figure 5. 3 and not determined). Since these prepatterning genes are required for the formation of SOPs, it is not clear whether the reduction in the levels of these patterning genes is resulting in a weaker ectopic SOPs phenotype or is contributing to the ectopic SOP phenotype seen upon *fred* knockdown. Interestingly, *stripe* whose expression is initiated by N signaling and positively regulated by Pnr is also decreased in expression in *fred* RNAi wing discs (Table 5. 2).

Klumpfuss (Klu), a Zinc finger transcription factor has been shown to be required for the formation of SOPs downstream to the proneural genes function. Mutations in *klu* do not decrease the expression of Ac/Sc in the SOP. Hence it is possible that Klu is required for maintainence of SOP fate after it has been determined (Klein and Campos-Ortega, 1997). It is not clear, how it’s decreased expression fits into the observed ectopic SOPs in *fred* mutants.

CG4427 is a predicted transcription factor with Zn finger and Sp-1 type motifs. An EP2237 line maps to this locus (Flybase). Misexpression of this gene mediated by the *pnr-GAL4* driver results in a deformed scutellum, extra scutellar macrochaeta and the microchaeta missing in the dorsal midline (Pena-Rangel et al., 2002). Misexpression with the *sca-GAL4* driver leads to formation of too many or too few scutellar or dorsocentral macrochaeta and frequent differentiation defects in the SOPs (Abdelilah-Seyfried et al., 2000). CG4427 is upregulated upon *fred* knock down. Based on the overexpression phenotype, it is possible that its increased expression, induced by *fred* knock down, is contributing to the *fred* RNAi phenotype.
CG8799 is predicted to encode an ATP-binding cassette transporter (Flybase). Interestingly, flies homozygous for an EMS induced mutation in this gene are semi-viable and show short bristles on the head, thorax and the scutellum (Personal communication to Flybase from K. Kozopas). CG8799 is upregulated in the absence of *fred*. It is not known whether overexpression of this transcript would affect SOP specification.

**Miscellaneous genes**

A number of known genes with no known roles in SOP specification are misregulated as are a number of predicted genes with no known function. Amongst these are transcription factors: Dorsocross3, defective proventriculus, apterous, spalt-major, pipsqueak and homothorax are downregulated upon *fred* knock down and transcription factor Sox100B is upregulated.

GTPase activating protein 1, Gap1, is a negative regulator of the EGFR signaling. EGFR signaling is transduced by the Ras/Raf/MAP kinase cascade. Gap1 inactivates RAS1 (RAS1 is activated by exchanging GDP for GTP) by stimulating its intrinsic GTPase activity (Gaul, 1992). It has recently been shown that EGFR signaling promotes the formation of SOPs and that an increase in EGFR signaling causes ectopic SOPs to arise (Culi et al., 2001). We have shown that Gap1 mutation enhances the *fred* RNAi rough eye phenotype in a dosage sensitive manner (Chandra et al., 2003). We have not examined how loss of one copy of Gap1 affects the ectopic SOPs phenotype induced upon *fred* knock down. Since increase in EGFR activity does not cause SOPs to arise in the non-neurogenic regions of the wing disc, decreased Gap1 activity, most probably will
not contribute to the specification of ectopic SOPs outside the proneural clusters in *fred* RNAi discs. Nevertheless, the observed genetic interaction between the two genes in the eye and the decreased levels of Gap1 in the *fred* RNAi wing discs strongly suggest a role of EGFR pathway in inducing Fred phenotype in the proneural clusters. Regulation of Gap1 expression has not been elucidated yet.

**Pnr cooperates with *fred* to suppress SOP formation in regions outside of the proneural cluster**

Knock down of *fred* leads to a reduction in the levels of *pnr*. Pnr is required for the formation of the DC and SC SOPs, decreased levels of Pnr result in lack of the DC and SC SOPs. *fred* knock down leads to formation of ectopic SOPs. In order to understand whether the decrease in *pnr* levels (in *fred* RNAi wing discs) has an effect on the ectopic SOP phenotype, we reduced the levels of *pnr* further. For this purpose, a combination of an amorphic mutation, *pnr*\textsuperscript{VX6} and a hypomorphic mutation, *pnr-GAL4* was used. Larvae of the genotype *pnr*\textsuperscript{VX6}/*pnr-GAL4: A101* lack the DC and SC SOPs (Figure 5. 4G). *pnr-GAL4* was also used mediate the expression of the *UAS-fred RNAi* construct. *UAS-fred RNAi; pnrGAL4: A101* larvae have ectopic SOPs (Figure 5. 4B and F). When *pnr* levels were further reduced in the *fred RNAi* background, an unexpected enhancement of the ectopic SOP phenotype was observed (Figure 5. 4D and H). This observation of reduction of *pnr* levels enhancing the ectopic SOP phenotype suggests that *pnr* is required to suppress SOP formation. However, in the *UAS-fredRNAi; pnr*\textsuperscript{VX6}/ *pnr-GAL4: A101* larvae, although ectopic SOPs are seen, the DC and SC SOPs are missing. Thus, *pnr* seems to have the role of promoting SOP formation in the DC and SC proneural
clusters, in addition to the role of suppressing their formation in the regions outside the proneural cluster (Figure 5.5). Another interesting aspect of pnr function is that reduction of pnr as in the pnrVX6/pnr-GAL4 wing discs does not result in the formation of ectopic SOPs (Figure 5.4C and G). It is possible that fred knock down causes an additional lowering of pnr levels which then results in ectopic SOPs. Alternatively, it is conceivable that pnr regulates certain negative regulators of SOP formation and that these regulators are redundant with the negative regulators activated by fred or are also activated by fred (Figure 5.5). Thus only when Fred is reduced along with Pnr, does the role of Pnr in suppressing SOP fate of the epidermal cells becomes apparent. Mosaic clones of cells null for pnr are usually very small as pnr is probably required for survival (Garcia-Garcia et al., 1999). However, they need to be studied to determine whether complete lack of pnr induces SOP cell fate in epidermal cells or it’s function of suppressing SOP fate is only required when Fred activity is also missing.

Using microarray analysis, I have determined a number of genes that are regulated by fred. By performing in situ hybridizations, I have shown that these changes in transcript levels can be validated in vivo. Many of the genes that are misregulated in fred RNAi wing discs, are in line with the observed phenotype and genetic interactions of fred. I have further examined the role of one of the genes, Pnr, that is down regulated upon fred knock down. My analysis of Pnr’s role in the context of Fred function has lead to the uncovering of a previously unknown activity of Pnr in suppressing SOP formation, in cooperation with Fred.
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Table 5.2: List of downregulated genes
Figure 5.1: Ectopic SOPs are induced upon *fred* knock down. X-gal staining of wing discs to visualize expression of lacZ in wing discs of the genotype (A) *Ap-GAL4/UAS-lacZ*, (B) *Ap-GAL4; A101*, (C and D) *Ap-GAL4; UAS-fredRNAi: A101*. A range of severity of the phenotype is obtained when Ap-GAL4 is used to mediate *fred* RNAi expression. Wing discs in (C) and (D) represent the weakest and the strongest phenotypes, respectively.
Figure 5. 2: Validation of upregulated genes. Control wing discs shown in (A-C) are of the genotype *Ap-GAL4; A101* and control wing disc in (D) is of the genotype *Ap-GAL4; Brd1.5-lacZ*. Experimental wing discs in (E-G) are of the genotype *UAS-fred RNAi; Ap-GAL4; A101* and experimental wing disc in (H) is of the genotype *UAS-fred RNAi; Ap-GAL4; Brd1.5-lacZ*. *In situ* hybridization was used to determine expression of transcripts CG10916, CG12505, CG3734 and X-gal staining was used to visualize expression of Brd. In the experimental wing discs (E-H) misexpression of each of the genes is apparent upon *fred* knock down. When *en-GAL4* driver that mediates expression only in the posterior region of the wing disc is used to express the *fred* RNAi construct, misexpression of these transcripts is limited to the posterior region (I-K).
**Figure 5.2:** Validation of upregulated genes.
Figure 5. 3: Validation of downregulated genes. *In situ* hybridization of control (*Ap-GAL4; A101*) wing discs (A, B, C) and experimental (*UAS-fred RNAi; Ap-GAL4; A101*) wing discs (D, E, F) using probes to recognize *pnr*, *mirr* and *E(spl)mβ*. A significant reduction in levels of these transcripts is evident in the experimental wing discs compared to the control wing discs.
Figure 5. 4: Genetic interaction between *fred* and *pnr*. X-gal staining for SOP marker, A101, in early third instar larvae (top panel) and late third instar larvae (bottom panel). Control wing discs of the genotype, *pnr-GAL4: A101* (A and E) show the normal pattern of SOPs. DC and SC SOPs in the late third instar larvae are visible (arrowheads). Upon *fred* knock down using the *pnr-GAL4* driver, ectopic expression of A101 is apparent (B and F; genotype *UAS-fred RNAi; pnrg-GAL4: A101*). *pnr*<sup>VX6</sup>*/pnrg-GAL4: A101* wing discs are shown in (C) and (G). Late third instar *pnr*<sup>VX6</sup>/ *pnrg-GAL4*wing discs have no DC and SC SOPs. A dramatic enhancement of the ectopic SOP phenotype seen in (B) is evident in (G), wing discs of the genotype *UAS-fred RNAi; pnrvx6/pnr-GAL4: A101*. *fred* knock down in the *pnrvx6* background also shows a much stronger ectopic SOP phenotype in late third instar larvae (H compare to F), although the enhancement is not as dramatic as in (D).
Figure 5.4: Genetic interaction between *fred* and *pnr*.
Figure 5.5: Model for roles of Pnr and Fred in suppressing SOP specification. Pnr positively regulates the levels of Ac/Sc in the DC and SC proneural clusters from which arise the DC and SC SOPs. Based on the enhancement of the *fred* RNAi phenotype, it can be proposed that Pnr inhibits SOP cell fate in the non neurogenic regions of the wing disc (regions where no proneural clusters are formed). However, no ectopic SOPs are induced in *pnrvx6/ pnrgAL4: A101* wing discs which suggests that this function of Pnr is redundant with that of Fred. It is possible that both Pnr and Fred upregulate inhibitors of *ac/sc* expression or function, leading to the suppression of SOP cell fate. It is also possible that Pnr, upon binding a transcriptional repressor, represses the expression of proneural genes. Fred might also be regulating *ac/sc* transcription.
ed and fred suppress neurogenesis but have spatially separable roles

Ed and Fred are paralogs that show an overall identity of 69% between their extracellular regions and an identity of 30% in the intracellular region. Both are expressed ubiquitously in the wing disc. Both ed and fred are required to suppress neurogenesis and they act non-redundantly in this process. However, the individual phenotypes of ed and fred in the wing discs are different.

Reducing Ed function in the wing disc using a temperature sensitive allele or by overexpressing a dominant negative construct results in formation of extra sensory bristles adjacent to the wild type bristle i.e. the extra bristles arise from the proneural clusters (Figure 3. 3E, 3. 4E and F). This observation points to a role of Ed in the process of lateral inhibition. Indeed, extra sensory bristle phenotype of ed can be compensated by increasing the activity of N signaling pathway (Figure 3. 6H) (Ahmed et al., 2003).

Another research group recently published a paper that has similar conclusions (Escudero et al., 2003).

Interestingly, extra SOPs are formed in fred RNAi wing discs not only in the proneural clusters but also in regions outside the proneural clusters (Figure 4. 6D, F, H and Figure 5. 1C). Thus, similar to Ed, Fred is required for lateral inhibition, but different
from Ed, Fred has an additional role of suppressing SOP formation in the non-neurogenic regions of the wing disc. Similar to ed, fred phenotype can be suppressed by increasing the activity of N signaling pathway (Figure 4. 7), implying the SOPs induced by lack of Fred function can be suppressed by ectopically increasing the N pathway which suppresses SOP formation (Chandra et al., 2003).

What makes Fred function different from Ed? Experiments to test whether Fred and Ed can rescue each other’s phenotype would help assign distinct functions to each protein that can further be dissected to test whether it is the intracellular or the extracellular domains that make these proteins function differently.

**ed and fred interact synergistically with the Notch signaling pathway**

Ed is required to suppress neurogenesis during CNS formation in embryos and PNS formation in larvae. Ed is also required to suppress wing vein specification (Figure 3. 1 and 3. 3A and B). A genetic epistatsis experiment was conducted to characterize interactions between ed and N pathway. ed mutant embryos show a hyperplasia of the nervous system. Expression of N^{act} suppresses neuronal specification. Embryos that are mutant for ed and are expressing N^{act} have a CNS showing a near wild type morphology. This observation of compensating as opposed to an epistatic phenotype suggest that during embryogenesis ed acts to inhibit neurogenesis through a pathway parallel to N signaling (Figure 3. 5A-H). In the wing disc, loss or reduction of ed causes extra wing vein material to form which is enhanced by reduction in N signaling (Figure 3. 5I-N). Interfering with Ed function by overexpressing a dominant negative construct of Ed, Ed^{Ext}, resulted in extra sensory bristles (Figure 3. 4D-G). This phenotype is enhanced by
overexpressing Dil that interferes with lateral inhibition (Figure 3. 6A-D). The EdEx phenotype is rescued by overexpressing E(spl)m7 or m8 (Figure 3. 6E-H and data not shown). When the transcript levels of genes of E(spl) complex was examined in ed mutants, a consistent change in levels of expression of these genes was not observed (data not shown). At present no targets of ed signaling have been identified. It is also not clear how the signal from ed is transduced to the nucleus. I have conducted experiments that show a requirement for the intracellular region of ed for its function, but at present no proteins interacting with this domain have been identified (Ahmed et al., 2003). The extracellular region of Ed has been shown to bind the L1-type cell adhesion molecule, Neurogian (Nrg). In the eye disc, Nrg has been shown to activate Ed in its function of antagonizing the EGFR pathway (Islam et al., 2003). It remains determined whether Nrg also acts a ligand for Ed in the wing disc during SOP formation.

In case of Fred, experiments involving overexpressing N, Su(H) showed substantial rescue of fred RNAi phenotype. Overexpression of E(spl)m7 or m8, almost completely rescue phenotypes associated with fred RNAi (Figure 4. 7). Numerous members of the E(spl)-C show a reduced expression in fred RNAi wing discs (Table 5. 2 and figure 5. 3). How is Fred affecting the read out of N activation? Does it enhance N and Dil binding? Does it stabilize N? Does it potentiate cleavage or release of the intracellular domain of N or does it affect a downstream member of the N pathway. It is also possible that Fred affects the transcript levels of E(spl)-C genes through a pathway different from N. No Fred interacting proteins have been reported so far.

Microarray analysis of fred RNAi wing discs has yielded a list of transcription factors whose expression depends on Fred function. It is not known whether these are
primary readouts of Fred signaling or the changes in their expression are a secondary consequence of Fred activity. Again, it is not clear how the signal is transduced from the membrane to the nucleus. It has also not been determined whether Nrg plays a role in Fred signaling.

Mechanism of Fred function in non-neurogenic regions of wing disc

The role of Fred in inhibiting neurogenesis in the regions outside the proneural cluster is novel for a gene involved in lateral inhibition. Loss of all the other genes that mediate lateral inhibition results in extra SOPs differentiating from the proneural clusters but not from cells in the non-neurogenic regions of the wing disc.

Microarray analysis points to a reduction in the N signaling as transducers of N activity, genes of the E(spl)-C were downregulated in fred RNAi wing discs (Table 5. 2 and Figure 5. 3). The reduction of E(spl)–C expression explains the lack of lateral inhibition in the proneural clusters, however, these genes are not required for suppressing neurogenesis outside of the proneural clusters (de Celis et al., 1996).

Microarray analysis also revealed that Brd expression has been upregulated (Table 5. 1 and Figure 5. 2). However, overexpression of Brd only causes extra SOPs to arise from the proneural clusters (Leviten and Posakony, 1996). Thus the overexpression of Brd would explain the lack of lateral inhibition but not the basis of origin of the ectopic SOPs outside of the proneural clusters.

Amongst the other genes known to have a role in SOP formation that were misregulated in fred RNAi wing discs, Pnr, Ara and Mirr (Table 5. 2 and Figure 5. 3), are intriguing. Pnr is required for SOP formation in the medial region; Ara and Mirr are
needed for SOP formation in the lateral region of the wing disc (Gomez-Skarmeta et al., 2003), via positive regulation of Ac/Sc expression. The expression levels of these genes is decreased in fred RNAi wing discs. Genetic interaction experiments suggest that Pnr cooperates with Fred to suppress SOP specification in the non-neurogenic regions of the wing disc (Figure 5. 4). The functions of Ara and Mirr with respect to Fred’s role in suppressing SOP formation need to determined.

When Ap-GAL4 is used to mediate fred RNAi construct expression, a big population of ectopic SOPs is induced in the wing pouch region (Figure 5. 1). At present no candidate genes are known that would explain the mechanism of Fred function in this region. However, some of the genes revealed by microarray analysis to be misregulated by fred knock down could be potential candidates. In situ hybridization for three predicted transcripts, CG3734 (a serine type peptidase), CG10916 (a ring finger protein) and CG12505 (a CCHC type Zn finger protein) showed that these transcripts are consistently and significantly misexpressed in wing pouch regions of fred RNAi wing discs (Figure 5. 2). It will be important to determine whether these genes play a role in attributing SOP fate to cell in the non-neurogenic regions of the wing disc.

**Summary of roles of Ed and Fred in SOP formation in the wing disc**

Role of Ed in the SOP specification in the wing disc is limited to the proneural clusters. In the proneural clusters, Ed is required for lateral inhibition as reducing Ed signaling (using ed<sup>ts</sup> allele) results in specification of extra sensory bristles in the vicinity of the normal sensory bristles (Figure 3. 3E and F). ed interacts synergistically with the N signaling pathway in this process (Figure 3. 6). As of now it is not clear if ed modulates
the activity of the N pathway by effecting any of the components of the pathway or has synergistic relations to the N pathway as it has the same function as N pathway but it is part of a different signaling system. The deduced function and interactions of Ed in the SOP specification in the wing disc has been summarized in Figure 6.1.

In this model, Fred is required to inhibit SOP specification in the proneural clusters and in regions outside it (Figure 4.6D, F, H and Figure 5.1C). Fred synergizes with the N pathway and is required for the expression of $E(spl)$-C genes (Figure 4.7, Table 5.2 and Figure 5.3). This interaction would explain the lack of lateral inhibition in the proneural clusters (Figure 6.1).

Fred positively regulates the levels of the patterning gene, pnr (Figure 5.3). Reducing Pnr function by itself does not lead to extra SOP specification. However, reducing pnr levels in the fred RNAi background prominently enhances the fred knock down phenotype (Figure 5.4). Thus, in the non-neurogenic regions of the wing disc, Pnr cooperates with Fred to suppress SOP specification (Figure 5.5 and 6.1).

In summary, my work has lead to a better understanding of the role of Ed in the process of neuronal specification and its genetic interaction with the N pathway. I have also identified a function of Ed paralog, Fred. I have established that similar to Ed, Fred inhibits neuronal specification and synergizes with the N pathway. In contrast to Ed, that apparently is not required to inhibit neurogenesis in the non-neurogenic regions of the wing disc, Fred is needed to suppress neuronal potential in these regions. Microarray analysis of fred knock down wing discs, revealed that amongst other genes, the prepatterm gene, pnr, is downregulated upon fred knock down. Genetic characterization of the role of Pnr in fred knock down wing discs revealed a novel function of Pnr. Pnr seems to be
cooperating with Fred in suppressing the neuronal potential of the non-neurogenic cells of the wing disc. Thus, further analysis of Fred function in relation to that of Pnr might elucidate mechanisms through which cell fates are assigned.
Figure 6. 1: Summary of roles of and genetic interactions of Ed and Fred in SOP specification in wing disc. Yellow circles indicate the non-neurogenic region of the wing disc, here normally no Ac/Sc is expressed and they have no neuronal potential. Red cells indicate the proneural cluster where Ac/Sc is expressed, endowing these cells with potential to develop into SOP (green cell). However, through the process of lateral inhibition mediated by the N pathway, only one or two SOP arise from the proneural cluster and the rest of the cells lose their neurogenic potential. Ed and Fred are both required non-redundantly for this process. It is not clear if Ed and Fred mediate lateral inhibition via interactions with the N pathway or through a separate pathway or both. In case of Fred, it is clear that Fred is required for expression of E(spl) genes, but whether it positively regulates E(spl) level by synergizing with N signaling or through another pathway is not known. Fred also positively regulates pnr levels. Pnr is required for the formation of proneural clusters. In the non-neurogenic region of the wing disc, Pnr is required to inhibit SOP formation when Fred is absent. Thus Pnr cooperates with Fred in suppressing SOP formation in the non-neurogenic regions of the wing disc.
Figure 6.1: Summary of roles of Ed and Fred during SOP specification in the wing disc.
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