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Non-redundant roles of E(spl) proteins during Drosophila neurogenesis

Jee-Eun Kim
West Virginia University

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**Non-redundant roles of E(spl) proteins during
Drosophila neurogenesis**

Jee-Eun Kim

Thesis submitted to the
**Eberly College of Arts and Sciences
at West Virginia University**
in partial fulfillment of the requirements
for the degree of

**Master of Science
in
Biology**

Ashok P. Bidwai, PhD, Chair
Clifton P. Bishop, PhD
Philip E. Keeting, PhD

Department of Biology

**Morgantown, West Virginia University
2008**

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Abstract

Non-redundant roles of E(spl) proteins during *Drosophila* neurogenesis

Jee-Eun Kim

The *Enhancer of split Complex (E(spl)C)* in *Drosophila melanogaster* encodes seven bHLH repressors that serve as the final effectors of the highly conserved Notch signaling pathway during the process of cell fate specification. These E(spl) proteins have been considered to be functionally redundant because of their structural similarity, and the observation that over-expression of individual E(spl) members leads to neural hypoplasia, in general. And additional compounding factor is that loss-of function alleles in individual E(spl) members have been unavailable. However, individual E(spl) members exhibit inordinate conservation through *Drosophila* evolution and are differentially expressed, findings which challenge the premise of functional redundancy. Thus, I have carried out studies to compare the roles of two E(spl) proteins, M5 and M8, during eye and bristle development, with emphasis on their phosphorylation by protein kinase CK2. The function of the phosphorylation domain and its influence on repression by M8 has also been assessed. These studies suggest that M8 and M5 elicit suppression of bristle development with different potencies, and that phosphorylation does not engender redundant outcomes on repression in vivo. Finally, a number of novel proteins have been identified based on their interaction with the phosphorylation domain of M8 in a yeast two-hybrid screen. In conclusion, these studies suggest that E(spl) proteins serve non-redundant and, perhaps, context-specific roles.

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List of Abbreviations

Wg/Wnt	Wingless
Hh	Hedgehog
TGF- β	Transforming Growth Factor-B
RTK/P	Receptor Tyrosine Kinase/Phosphatase
N	Notch
DI	Delta
Ser	Serrate
Su(H)	Suppressor of Hairless
bHLH	basic Helix-loop-helix
<i>E(spl)C</i>	<i>Enhancer of split</i> Complex
CtD	C-terminal domain
N ^{ICD}	Notch intracellular domain
Dm	<i>Drosophila melanogaster</i>
IOB	Interommatidial bristle
ASC	<i>Achaete-scute</i> Complex
Gro	Groucho
Ato	Atonal
<i>E(spl)^D</i>	dominant allele of <i>m8/E(spl)</i>
Sca	Scabrous
Dpp	Decapentaplegic
H	Hairy

Chapter 1

General Introduction

1. Cell Fate Determination

One of the key processes during the development of an organism is cell fate determination. A simple, one-cell zygote develops into a multi-cellular organism in which previously undifferentiated cells take on specific functions and organize into complex structures through cell fate determination. The achievement of a desired harmony in such complexity depends on cross talk between cells, which involves signaling pathways that synchronize both inter- and intra-cellular communications. Examples of such signaling pathways include Wingless (Wg/Wnt), Hedgehog (Hh), Transforming Growth Factor- β (TGF- β), Receptor Tyrosine Kinase/Phosphatase (RTK/P), and Notch (N). These signaling pathways are conserved throughout metazoan taxa, from insects, amphibians to mammals. The coordinated activities of these pathways bring about differential gene expression in the regulated cells to initiate and maintain cellular differentiation during embryonic and post-natal development.

Proper formation and function of an organism thus depends on the precise execution of the signaling pathways, and disturbance or mutation in the pathways can result in developmental defects or disorders. In humans, for example, dysfunction in the Notch pathway is implicated in many cancers (Allenspach et al., 2002), leukemia (Jundt et al., 2008), multiple sclerosis (John et al., 2002), strokes/dementia (Joutel et al., 1996), and congenital heart diseases (High and Epstein, 2008; Watanabe et al., 2006). Understanding how the signaling pathways work should enhance our ability to better diagnose and treat such developmentally linked diseases and disorders (Nickoloff et al., 2003). This introduction concentrates mainly on the role of Notch signaling during *Drosophila* development as is relevant to the studies described within this thesis.

2. *Drosophila* as a Model Organism

The use of *Drosophila melanogaster* as a model organism came about when Thomas Hunt Morgan used the mutations in *Drosophila* to demonstrate genes located on chromosomes as the mechanistic basis of heredity, for which he was awarded the Nobel Prize in 1933. His work also laid the foundation for much of the modern genetics. The discovery of homeobox by Edward B. Lewis was came about when he described the *ultrabithorax* (*Ubx*) mutation in *Drosophila*, which results in a total transformation of one body segment with the other. These studies first revealed the merit of using fruit fly as a model system to study developmental genetics. Subsequently, Christiane Nusslein-Volhard and Eric Weischaus conducted a systematic investigation to identify genetic controls during early embryonic *Drosophila* development (Nusslein-Volhard and Weischaus, 1980). Their seminal work further established *Drosophila* as a model organism to study genetics, and especially development biology.

Drosophila has many advantages over other model organisms such as mouse, earthworm or zebrafish that have been used to understand genetic regulations during development. The followings are some of the highlighted reasons behind the popularity and suitability of fruit fly as model system. Small size and short generation period is ideal to conduct large-scale genetic studies in relatively short time periods in limited laboratory space. The low cost and ease of maintaining the flies also attract scientists. The numerous molecular and genetic tools and techniques, the breadth of accessible transgenic fly stock resources, an extensive research community and network, and the completely sequenced genomes of several species of *Drosophila* broaden the kinds of experiments that can be performed with fruit flies. In addition, its high homology and the application of findings to mammalian system, while avoiding many ethical concerns concordant with vertebrate research, ease the research process.

With the completed genomic sequence analyses of human, mouse and fruit fly, it is ever more apparent that the key genetic controls during development of mammals and insects are conserved. Genetic studies conducted in *Drosophila* have led to identification of genes and their regulatory actions during various developmental stages. Many of the genes discovered to be important during *Drosophila* development have also been investigated in mammalian systems to ultimately enhance our understanding of the pathology of genetically-linked diseases and disorders in human.

3. The Notch Pathway

Notch signaling is a highly conserved pathway that is evoked during localized cell-cell communications in all metazoan organisms. In general, the Notch pathway restricts a specific cell fate to one cell from a group of equipotent cells by controlling cellular differentiation, proliferation and apoptosis. Its involvement in developmental processes includes neurogenesis, myogenesis, and oogenesis (for reviews, see (Artavanis-Tsakonas et al., 1999; Mumm and Kopan, 2000)).

The role of Notch pathway has been studied extensively in different developmental contexts of various organisms. The two such contexts are the neural development of the compound eye and the mechano-sensory bristles in *Drosophila*. In fact, the gene *notch* was first discovered in *Drosophila* by Thomas Morgan who named the gene according to the 'notch' phenotype elicited on the wing margin. To a large extent, what has been learned about the Notch signaling in these two developmental systems has served as a paradigm to understand its roles in other contexts. In addition to *Drosophila* neurogenesis, the Notch pathway controls cell lineage and germ-line proliferation during vulval development in the nematode *Caenorhabditis elegans* (Greenwald, 1998; Greenwald, 2005). Vascular

Table 1-1: Core components of the Notch signaling cascade

	<i>D. melanogaster</i>	<i>C. elegans</i>	Mammals
Receptor	Notch	LIN-12 GLP-1	Notch1 ~ Notch4
Ligand	Delta Serrate	APX-1 LAG-2	Delta1, 2 Jagged 1, 2
Transcription factor	Su(H)	LAG-1	CBF1/RJBk
Transcription factor	E(spl) bHLH proteins	-----	Hairy/E(spl) (HES)
Co-repressor	Groucho	-----	TLE

The major components of the Notch pathway are highly conserved from *D. melanogaster*, *C. elegans* to mammals. The role of Notch is also analogous in three organisms in that it governs cell fate specification at different stages of development.

development and homeostasis in vertebrates are also controlled by Notch signaling (Roca and Adams, 2007; Uyttendaele, 2001).

As in other organisms, this pathway in *Drosophila* includes the membrane-associated receptor Notch (N), its ligands Delta (DI) or Serrate (Ser), its downstream component Suppressor of Hairless (Su(H)), and the final effectors the basic Helix-loop-helix (bHLH) transcription repressors which are encoded by the Enhancer of split Complex (E(spl)(C)) (Greenwald, 1998). Both the receptor and ligand proteins are single-pass transmembrane proteins, which necessitates presence of both components to be on the plasma membrane of adjacent cells for interaction. The core elements of the Notch pathway from three well-studied organisms are listed in Table 1.

4. Notch Signaling during *Drosophila* Neurogenesis

The Notch pathway acts in a paracrine manner between adjacent cells that possess the same developmental potency (Figure 1-1). In the case of *Drosophila* neurogenesis, the Notch pathway confines the development of one sensory organ precursor (SOP) in the case of bristle development, or R8 in the case of eye development, from a group of equipotent cells that is also referred to as a proneural cluster (PNC). The cell destined to become a neuronal cell expresses the ligand Delta/Serrate, which binds to the receptor Notch on the adjacent cells within the PNC. Ligand-receptor interactions activate the Notch pathway in the cells receiving this signal, and result in the cleavage and the release of Notch intracellular domain N^{ICD} (Schroeter et al., 1998). This fragment translocates into the nucleus where it converts the transcriptional repressor Su(H) into an activator. Subsequently, Su(H) mediates the expression of the E(spl) repressors (Bailey and Posakony, 1995; Lecourtois and Schweisguth, 1995) that antagonize the activity of the proneural activators required for specification of the neuronal fate.

4.1. Notch signaling during eye development

The compound eye of *Drosophila* comprises approximately 800 light-sensing facets called ommatidia. Each ommatidium contains 20 cells: 8 photoreceptor neurons (the retinula cells R1-R8), 12 accessory cells including the cone cells that form the lens, and the primary and secondary pigment cells that optically insulate ommatidia from each other (for reviews, see (Freeman, 1997)). These ommatidia are arranged in a precise hexagonal lattice, wherein interommatidial bristles (IOB's) are specified at alternating vertices of each ommatidium. Even a subtle perturbation in cell fate specification can disrupt this hexagonal patterning and phenotypically manifest in the adult fly eye. Thus, the unique two-dimensional crystalline arrangement of the compound eye provides an excellent model to understand the mechanisms by which these cell fates are assigned and the underlying cell signaling events.

The development of all cell types that comprise a complete ommatidium primarily depends on the specification of the R8 cells, which are therefore referred to as the 'founding' photoreceptors. The R8 cells orchestrate ommatidial assembly, and their positional specification in the developing retina is crucial for establishing the final architecture of the adult eye. Once the R8 cells have been specified, they subsequently recruit the neighboring cells (Jarman et al., 1994) to adopt the remaining photoreceptor cell fates (R1–R7) in an invariant order of R2/R5, R3/R4, R1/R6 and finally R7 fates. Because the R8's perform this critical function, substantial efforts have been devoted to unravel the biochemical events that mediate their specification.

4.2. R8 specification

The specification of R8 cells occurs in the eye imaginal disc of third instar larvae in a wave of differentiation known as the morphogenetic furrow (MF) that sweeps across the eye

Figure 1-1: Notch pathway

The Notch pathway restricts a specific cell fate to a single cell from a group of equipotent cells. The diagram illustrates the Notch pathway during sensory organ precursor (SOP)/R8 selection from proneural cluster cells (PNC) in *Drosophila* neurogenesis. SOP/R8 restrains the neighboring cells from adopting its neuronal fate by expressing the ligand Delta to send the inhibitory signal through the receptor Notch on the neighboring cells. Activated Notch undergoes proteolysis to release its intracellular domain (N^{ICD}), which translocates to the nucleus to induce Su(H)-dependent expression of E(spl) bHLH proteins. E(spl) proteins then repress cells receiving the inhibitory signal from adopting neuronal fate.

disc from its posterior to anterior margin (for reviews, see (Frankfort and Mardon, 2002)). In its wake, the MF leaves behind specified R8 photoreceptors that are positioned in an alternating lattice. R8 specification, which requires the activity of proneural activator Atonal (Ato), involves the Notch signaling pathway in a biphasic manner (Baonza and Freeman, 2005; Li and Baker, 2001). Initially, Notch establishes Ato expression (proneural Notch signaling), but in subsequent stage Notch inhibits Ato expression (inhibitory Notch signaling).

The process of R8 specification is divided into four stages (Figure 1-2 and Figure 1-3). Notch augments *ato* expression first initiated by Hedgehog and Decapentaplegic (Greenwood and Struhl, 1999; Sun et al., 1998) to accumulate in clusters of cells along the D-V axis of the eye disc (Baker et al., 1996; Baker and Yu, 1997) by relieving the Su(H)-mediated repression at the anterior margin of the MF (Li and Baker, 2001). This process is termed 'proneural enhancement' (Stage 1). Once Ato starts to accumulate, *ato* expression is maintained by the auto positive-feedback loop, in which Ato protein activates its own transcription (Baker et al., 1996). The clusters of cells expressing *ato* are akin to the proneural clusters (PNC) seen in other neurogenesis, for they all have the potential to become R8 cells as long as they maintain *ato* expression (Jarman et al., 1995; Jarman et al., 1994). However, not all the cells from each PNC become the R8 cell. When one cell from a PNC gains an advantage over the others, in the sense that it expresses the highest level of Ato, this pro-R8 cell initiates the Notch-dependent lateral inhibition process (Stage 2/3) (Sun et al., 1998)

During lateral inhibition (Stage 2/3), unlike in the 'proneural enhancement' (Stage 1), Notch signaling leads to the suppression of *ato* expression in the Notch activated cells. Specifically, the future R8 cells begin to express the ligand Delta which activates Notch receptor in immediate neighboring cells. Activation of Notch leads to its cleavage and the release of intracellular domain N^{ICD} in the activated cells (Schroeter et al., 1998). This N^{ICD}

Figure 1-2: Notch signaling during R8 specification

Notch (N) plays biphasic roles during R8 specification. N establishes Atonal (Ato) expression during 'neural enhancement' (Stage 1) in proneural clusters (PNC) prior to the morphogenetic furrow (MF). In contrast, during lateral inhibition, N signaling restricts the Ato expression to a single cell (R8 cell) within each PNC (Stage 2/3/4). Notch signaling is also involved during specification of secondary photoreceptors, which is initiated by the specified R8 cells.

fragment translocates to the nucleus and converts the transcriptional repressor Su(H) into an activator. Subsequently, Su(H) mediates the expression of the E(spl) repressors that antagonize the activity of Ato to disrupt the auto positive-feedback loop of *ato* transcription (Bailey and Posakony, 1995; Ligoxygakis et al., 1998).

Refinement of PNC into a single R8 cell is also thought to involve a feedback loop within the Notch signaling that reinforces the lateral inhibition. (Figure 1-3) At the beginning of lateral inhibition, all precursors express low level of both Notch and Delta, which means that they all have the ability to inhibit each other from becoming the R8 cell. However, when one cell (R8 precursor) within the PNC begins to express higher level of Ato, this stochastic difference is thought to be amplified by a negative feedback loop between Notch and Delta (Heitzler et al., 1996). As the feedback loop persists, R8 precursor cell becomes more potent at inhibiting neighboring cells. On the other hand, neighboring cells receive greater magnitude of Notch inhibitory signal, which represses ligand expression in them (Heitzler et al., 1996). Genetic evidence also raise the possibility of additional machineries involving other signaling pathways, possibly EGFR, which predispose a certain cell(s) within the PNC to be R8 precursor (Dokucu et al., 1996).

As the result of lateral inhibition, cells that receive the inhibitory Notch signaling rapidly lose *ato* expression, and are thus redirected away from adopting the (default) R8 fate (Stage 4). These neighboring cells are then recruited by the R8 cells to undergo secondary cell fate specification (Jarman et al., 1994). Notch is also required during some of these subsequent steps of ommatidial assembly. For example, Notch and its final effector E(spl) proteins are required for the specification of the R3/R4 (Fanto and Mlodzik, 1999).

Although unique to eye development, the model of biphasic Notch function during R8 specification is consistent with the experimental findings. Loss of Notch at the first phase of the specification leads to loss of *ato* expression and abolition of eye development,

Proneural Enhancement (Stage 1)

Lateral Inhibition (Stage 2/3)

R8 Specified (Stage 4)

Figure 1-3: Molecular events during R8 specification

During proneural enhancement (Stage 1), all cells in PNC express *Ato*. Notch initiates the *ato* expression by relieving repression by Su(H). When one (pro-R8 cell) expresses the highest level of *Ato*, it initiates the process of Notch-dependent lateral inhibition (Stage 2/3). Pro R8-cell expresses ligand Delta, which binds Notch receptor on neighboring cells. This interaction causes the hydrolysis of Notch intracellular domain (N^{ICD}), which translocates into nucleus to initiate transcription of *E(spl)*/*C* genes by converting Su(H) into an transcription activator. This lateral inhibition by pro-R8 cell is amplified by a feedback loop to achieve a single R8 cell from each PNC.

which is phenotypically similar to flies carrying the null allele *ato*¹ (Jarman et al., 1994; Ligoxygakis et al., 1998). In contrast, loss of Notch during the second phase compromises lateral inhibition, leading to the specification of supernumerary R8 cells (Ligoxygakis et al., 1998). Further evidence in favor of this model is the observation that loss of Su(H) does not affect *ato* expression, but attenuates E(spl) expression which also results in aberrant lateral inhibition (Koelzer and Klein, 2003).

5. Notch Signaling during Bristle Development

The Notch signaling also plays a key role in mechanosensory bristle development (Hartenstein and Posakony, 1990; Modolell and Campuzano, 1998). The notum of *Drosophila* is characterized by the presence of 22 macrochaetes (large bristles), which are mechanosensory organs of the peripheral nervous system. Each bristle consists of four cell types that arise from a single precursor cell known as sensory organ precursor (SOP). The SOP is akin to the R8 cell in the retina. The specification of the SOP's occurs in the third larval instar, but they do not undergo morphogenesis until the pupal stage. In the third larval instar, proneural clusters (PNCs) are formed at precise positions in the wing imaginal disc, which is the primordial tissue from which the adult thorax arises. The formation of these PNC's requires the proneural bHLH transcription activators encoded by the *achaete-scute Complex* (ASC) (Cubas et al., 1991). Unlike the R8 specification, the initial ASC expression in PNC is under the control of pre-pattern factors and does not involve Notch (Calleja et al., 2002). Only the inhibitory aspect of Notch is involved during bristle development.

When one cell from a PNC gains an advantage over its neighbors, in that it expresses the highest level of ASC, it initiates the process of Notch-dependent lateral inhibition whose molecular determinants are virtually identical to those during the R8

refinement (Castro et al., 2005; Giebel and Campos-Ortega, 1997) (Figure 1-4). As a result, a single SOP arises from each PNC. This SOP then undergoes two asymmetric divisions that are also dependent upon Notch and E(spl). The SOP first divides to give rise to two 2nd order precursors known as pIIa and pIIb. The pIIb prevents pIIa from taking on the pIIb (default) fate by expressing Delta which activates Notch on pIIa. Asymmetric division of pIIa gives rise to the socket and shaft cells, whereas pIIb gives rise to a glial cell and a 3rd order precursor, pIIIb. Again, the shaft cell inhibits the socket cell from taking on the shaft (default) fate through the inhibitory Notch signaling. Subsequently, asymmetric division of the pIIIb cell gives rise to the sheath and neuronal cells, in which the neuronal cell prevents the sheath from taking the neuronal (default) fate through Notch-Delta interaction.

Evidence in support for role of Notch during bristle morphogenesis is the observation that loss of Notch during SOP selection leads to supernumerary SOP's that manifest in the adult fly as closely juxtaposed ectopic bristles (Hartenstein and Posakony, 1990). In contrast, loss of Notch after SOP selection has been shown to skew the asymmetric divisions. For example, loss of Notch during the first asymmetric division of the SOP leads to the specification of four neuronal cells as a result of all daughter cells automatically assuming the default (neuronal) fate, which manifests as missing bristles (Hartenstein and Posakony, 1990).

Figure 1-4: Notch signaling during SOP development

The inhibitory Notch (N) signaling is required for the selection of a single SOP from a proneural cluster (PNC). It is also involved during the subsequent steps of SOP divisions which give rise to four sister cells that make up a mechanosensory bristle. The lateral inhibition during SOP development is analogous to that seen during R8 specification, and is mediated by receptor Notch, ligand Serrate, Su(H) and E(spl) repressors.

6. E(spl)C Repressors in Notch Signaling

As the final downstream effectors of the Notch signaling, the E(spl) repressors are critical for proper neural development. The *E(spl)C* in *Drosophila* is located on the 3rd chromosome, and encodes seven bHLH proteins (MA, MB, MC, M3, M5, M7, and M8), and the non-bHLH protein Groucho (Gro) (Delidakis et al., 1991; Knust et al., 1992) (Figure 1-5A). All seven bHLH proteins appear similar in overall architectures, and contain a number of functional motifs common to all of them (Giebel and Campos-ortega, 1997) (Figure 1-5B). These functional domains are in order from the N-terminus: a basic domain, an HLH motif, a second HLH motif termed the Orange domain, and a WRPW tetrapeptide located at the C-terminus. It has been shown that the basic domain is required for DNA binding, and recognizes a unique sequence (CACNAG) referred to as the N-box (Tietze et al., 1992). The first HLH motif is required for homo/heterodimer formation between E(spl) repressor members, whereas the second HLH motif mediates interaction of E(spl) proteins with proneural activators ASC/Ato. The C-terminal WRPW motif, which is invariant among all E(spl) members and also found in Hairy and Deadpan (two other Notch-responsive proteins found in other locus) (Maier et al., 1993), recruits the obligatory co-repressor Gro (Paroush et al., 1994).

Given the importance of E(spl) proteins during Notch-mediated lateral inhibition, substantial efforts have been directed at defining the mechanisms by which they mediate neural repression (Figure 1-3). These studies have exploited the observation that ectopically expressed E(spl) proteins potently repress neural development (Nakao and Campos-Ortega, 1996). It was initially thought that E(spl) proteins mediate neural repression by inhibiting transcription of proneural genes such as *ASC/ato* through DNA-binding (Van Doren et al., 1994). This model arose because an E(spl)-specific DNA-binding element named the N-box was found in the enhancer of *ASC* (Oellers et al., 1994).

A. *E(spl)C* locus

B. Functional domains in *E(spl)* proteins

Figure 1-5: *E(spl)C* locus and functional domains in *E(spl)* proteins

(A) *E(spl)C* locus encodes seven bHLH proteins and one non-bHLH protein Groucho (Gro).
 (B) *E(spl)* proteins share four functional domains. Basic domain is required for DNA binding. The first HLH motif is required for homo/heterodimer formation between *E(spl)* members, while the second HLH motif (Orange) mediates interaction of *E(spl)* proteins with proneural activators ASC/Ato. The C-terminal WRPW motif recruits the obligatory co-repressor Gro.

This, however, turns out not to be the case. For example, deletion of the N-box in the ASC enhancer or mutations that abrogate DNA-binding by E(spl) proteins do not compromise lateral inhibition (Nakao and Campos-Ortega, 1996). In addition, no N-box like sequence has so far been identified in the *ato* enhancers.

The more prevailing model, supported by a growing body of evidence, is that E(spl) proteins are likely to function by directly binding and inhibiting the activities of proneural activators through their Orange domain (Dawson et al., 1995; Giebel and Campos-ortega, 1997). This model, termed the 'protein-tether', is based on genetic data that describe the mechanism by which proneural activators promote their own transcriptions, and the observed direct protein interactions between proneural activators and E(spl) repressors in the yeast two-hybrid and protein-based assays (Alifragis et al., 1997; Gigliani et al., 1996). Studies in the eye, particularly with regards to the direct protein antagonism of Ato by E(spl) proteins have provided the strongest evidence for the underlying mechanism (Figure 1-3). During R8 selection, the early expression of *ato* initiated by Notch in cells at Stage 1 (proneural enhancement) of the MF is mediated through the activity of the 3' enhancer whose activity is independent of endogenous Ato levels (Baker et al., 1996). Subsequently in Stage 2/3 (lateral inhibition), function of the Notch signaling switches from proneural to inhibitory, and the activity of the 3' enhancer also discontinues. Rather, Ato concentrations rise to high levels because of the activity of the 5' enhancer that responds to the accumulated Ato protein through a consensus binding site (E-box, CACGTG) found in Ato (Sun et al., 1998). In this context, E(spl) proteins antagonize the second phase of *ato* expression by dominantly squelching the Ato-dependent auto-regulatory feedback loop. It is this squelching that requires the direct binding between proneural activators and E(spl) proteins.

7. Scope of Thesis

The thesis consists of three research projects, thus each chapter focuses on results and discussions from each project. Although three projects have distinctive characteristics, they have been arranged in the order as to give the best continuum to the overall implication of the thesis, which suggests non-redundant roles of E(spl) proteins during *Drosophila* neurogenesis.

Chapter 2

This chapter presents the mechanism of post-translational regulation of E(spl)M8 during *Drosophila* development. Genetic data from our laboratory show that phosphorylation by protein kinase CK2 is required for repression by M8 during R8 specification. In comparison to previous studies of a dominant allele of *m8* (*E(spl)^D*), the CK2 phosphorylation is thought to engender a conformation change that overcomes an auto-inhibited state of M8. This auto-inhibition is thought to involve the C-terminal domain (CtD), which directly/indirectly occludes the Orange domain. In this model, the CtD restrains M8 by blocking the ability of Orange domain to mediate interaction with proneural activators such as ASC and Atonal. This mechanism has been investigated by a direct genetic approach. Constructs containing only the CtD of full-length M8 (M8-CtD) or M8SD (M8SD-CtD) were over-expressed to assess if the CtD fragment could affect bristle and/or retinal patterning by inhibiting (in trans) the endogenous M8 activity.

Chapter 3

There are seven bHLH repressors encoded by *E(spl)C* which are thought to be functionally redundant because of structural similarity and the lack of individual loss-of-function alleles. In vitro studies indicate that CK2 also phosphorylates M5 and M7, in addition to M8. I have employed molecular and genetic approaches previously used to

assess the effects of M8 phosphorylation to investigate the functional significance of M5 phosphorylation by CK2, and to assess whether E(spl) proteins overlap in their functions during *Drosophila* neurogenesis.

Chapter 4

The CK2 recognition motif in E(spl)M5/7/8 is preceded by another highly conserved sequence, which our laboratory calls the 'SPASSGY' motif. This sequence is also conserved in M8 homologs in other *Drosophila* species and mammals. The SPASSGY site contains highly conserved Ser/Tyr residues that may be targets for secondary phosphorylation by kinases other than CK2. In order to identify secondary interacting partners of phosphorylated M8, a yeast two-hybrid screen of the embryonic *Drosophila* cDNA library has been conducted using M8SD-CtD as bait.

Chapter 2

The C-terminal domain of E(spl)M8 regulates repression activity of M8.

1. Abstract

This chapter focuses on the mechanism of post-translational regulation of E(spl)M8 during *Drosophila* neurogenesis. Genetic data from our laboratory show that phosphorylation by protein kinase CK2 negatively controls the repressor activity of M8 during R8 specification. Comparison to previous studies on a dominant allele of *m8* (*E(spl)^D*) indicates that this negative control of M8, i.e. auto-inhibition, may involve the internal C-terminal domain (CtD), which encompasses the region after the Orange domain. The CtD region is thought to restrain M8 from exerting neural repression by blocking the interaction of M8 with proneural activator Atonal. I have investigated the CtD-mediated auto-inhibition hypothesis by employing a direct genetic approach. Constructs containing only the CtD of the full-length M8 (M8-CtD) or M8SD (M8SD-CtD) were over-expressed using transgenic flies to analyze if the CtD fragment is indeed the mechanistic basis of auto-inhibition, and whether this cis-regulation is CK2-phosphorylation dependent. The results show that ectopically expressed M8-CtD suppresses endogenous M8 activity. However, M8SD-CtD also appears competent at mediating such effect. The possible reasons are discussed.

2. Introduction

The final players of the Notch signaling cascade are bHLH repressors encoded by *E(spl)C* located on the third chromosome of *Drosophila*. The locus encodes seven such proteins, which are structurally homologous, but it is still unclear whether they play redundant/overlapping functions as part of Notch signaling. The diverse involvement of Notch signaling during development has raised the possibility that these repressors are differentially employed in a context-specific manner. Loss- and gain-of function studies as well as analysis of expression patterns support this possibility. For example, the seven *E(spl)* members exhibit non-overlapping expression patterns during larval development, and misexpression of individual *E(spl)* members elicits dominant neural defects that are isoform-specific. The model of functional specialization of *E(spl)* proteins is gaining momentum as data accumulate. (This topic will be discussed further in Chapter 3). Distinct expression patterns of each member also suggest the existence of additional controls. One such mechanism could be post-translational regulation of the repressors. It is common that a protein is first translated in an inactive form, and then activated by post-translational modifications such as phosphorylation, cleavage or splicing. Work from our laboratory suggests that the activity of *E(spl)M8* repressor is regulated post-translationally via phosphorylation by protein kinase CK2. This phosphorylation event appears to augment the potency with which M8 mediates neural repression in vivo.

2.1. Protein kinase CK2

CK2 is a ubiquitously expressed protein kinase that specifically phosphorylates Ser/Thr residue on target proteins (Glover, 1998; Pinna, 1994). It is one of the most extensively studied kinases, and is known to be involved in cellular processes such as cell cycle progression, DNA replication, transcription, translation, cell polarity, and development

(Litchfield, 2003; Meggio and Pinna, 2003). In fact, the number of its potential targets is predicted to be 10% of all the cellular/organismal phosphoproteome. In *Drosophila*, it is a tetrameric holoenzyme composed of two catalytic alpha subunits and two regulatory beta subunits (Glover et al., 1983). The holoenzyme shows high substrate specificity, which is achieved by recognizing a consensus sequence, (S/T)(D/E)x(D/E) (Kuenzel and Krebs, 1985; Kuenzel et al., 1987). In this motif, the acidic residues at positions n+1 and n+3 play a critical role in the phosphotransferase reaction. This preference for acidic microdomains is unique to CK2 among virtually all members of the Ser/Thr protein kinase family. A catalytic subunit monomer (CK2 α) also displays substrate specificity that is equivalent to that of the holoenzyme, suggesting that substrate specificity determinants are intrinsic to CK2 α (Bidwai et al., 1993).

2.2. CK2 Phosphorylation of M8

Biochemical Analysis

The interaction between E(spl)M8 and CK2 α was identified by our laboratory during a large-scale two hybrid screen for *Drosophila* embryonic cDNA's whose products interacted with *Drosophila* CK2 α (DmCK2 α) (Trott et al., 2001a). The screen identified and characterized several potential targets of CK2 including E(spl)M7. The structural similarities between E(spl) proteins led to follow-up studies to investigate whether CK2 α interacts with other E(spl) members (Trott et al., 2001b). The two-hybrid approach revealed that three E(spl) proteins, M5, M7 and M8, but not other E(spl) proteins, interact with CK2 α . When sequences of the seven E(spl) proteins were aligned, the CK2 recognition motif, S(E/D)x(E/D), was found only in these three E(spl) proteins (M5, M7 and M8). Prior studies had identified four functional motifs were shared amongst all seven E(spl) members, but the discovery of the CK2 recognition consensus was novel. The CK2 consensus site was found

in the C-terminus domain (CtD), between the Orange and WRPW domains. This finding revealed that a subset of E(spl) repressors contain an additional motif, i.e. the CK2 recognition sequence. Phosphorylation assays of purified GST-tethered M5, M7 and M8 with either holoenzyme CK2 or CK2 α subunit alone showed that the CK2 site in these E(spl) proteins was indeed biologically functional. Efforts to map the phosphorylation site in M8 made use of CK2-variants of M8 that harbor a substitution at Ser-159, which is the first Ser residue in the CK2 consensus motif. M8SD substitutes Ser-159 with Asp to mimic the constitutively phosphorylated state, while M8SA replaces Ser-159 with Ala to mimic the non-phosphorylatable state. Only the wild-type M8 was phosphorylated, while M8SD or M8SA were not, which confirmed Ser-159 as the only possible site of CK2 phosphorylation.

Genetic Analysis

Of three E(spl) proteins, M8 was chosen for genetic analysis because an allele of M8 (*E(spl)^D*) has been implicated in *Drosophila* eye development. Transgenic flies expressing M8 and its CK2 variants were generated to investigate the functional significance of M8 phosphorylation in vivo. Ectopic expression of M8SD resulted in a severely reduced eye phenotype as a consequence of reduced number of specified R8's during lateral inhibition (Karandikar et al., 2004). On the other hand, ectopic expression of M8 or M8SA did not elicit any such phenotype. The reduced eye of M8SD was of interest because of its similarity to the retinal defects of *N^{spl}/Y; E(spl)^D/+* flies, which have been known for many years (Nagel et al., 1999). *E(spl)^D* is a unique dominant allele of *m8* (the gene *m8* was originally named *E(spl)* when it was discovered) that was identified based upon its genetic interaction with the recessive Notch allele, *N^{spl}* (Welshons, 1956). *E(spl)^D* has a mutation in the *m8* transcription unit that introduces a stop codon immediately after the Orange domain. As a result, the mutant protein, called M8*, lacks all the C-terminal sequences after the Orange domain including the CK2 recognition motif and the WRPW domain (Klamt et al., 1989; Tietze et

al., 1992). *E(spl)^D/+* flies display no overt eye defects (Campos-Ortega and Knust, 1990), but when combined with *N^{spl}* exhibits a severely reduced eye phenotype (Nagel et al., 1999). The effect of M8* was attributed to its accentuated activity against Ato, as reflected in in-vitro protein interaction assays (Nagel and Preiss, 1999). A similar affinity for Ato was also observed with M8SD, but not with wild-type M8 (Karandikar et al., 2004). The dominant effect of *E(spl)^D* has, however, remained enigmatic. For example, loss of the WRPW sequence neutralizes repression by all E(spl) members, a property that is also shared with the related bHLH repressors Hairy and Deadpan (Fisher and Caudy, 1998; Paroush et al., 1994). Thus, *E(spl)^D* has been described as a Gro-independent hypermorph (Nagel et al., 1999). Based on the similar retinal defects and Ato-interaction between M8SD and M8*, the possibility arises that the C-terminal region may have a regulatory function that restrains M8 activity. This C-terminal auto-inhibitory region is missing in M8*, thus predisposing this protein to precocious repressor activity.

2.3. Proposed auto-inhibition model

The similar reduced eye phenotype of M8SD to that of M8* gave some insight into the *N^{spl}/Y; E(spl)^D/+* phenotype. As mentioned above, M8* leads to greater neural suppression during R8 specification because it displays an accentuated interaction with Ato. M8SD also displays higher interaction with Ato, which suggests that a similar mechanism appears to underlie the reduced eye phenotype of M8SD. However, M8SD still retains the C-terminal region missing in M8* that is speculated to exert an auto-inhibitory regulation, and also has a substitution of Ser-159 with Asp to mimic the phosphorylated state. In fact, the interaction between the full-length M8 (without phosphate group at Ser-159) and Ato is marginal in vitro (Karandikar et al., 2004).

These findings suggested that M8 may exist in two conformations with the phosphorylation functioning as a switch (Figure 2-1). In a non-phosphorylated state, M8 is

Figure 2-1: Auto-inhibition model

M8 may exist in two conformations with the phosphorylation functioning as a switch. The C-terminal domain (CtD) of M8 is thought to occlude the Orange domain from binding the proneural activators such as Atonal/ASC, thus restraining its repressor activity ('auto-inhibition'). This auto-inhibition by the CtD is relieved when CK2 phosphorylates M8, which is thought to engender a conformation change to displace the CtD from the Orange domain.

restrained from binding Ato because its Orange domain is occluded by the C-terminal domain (CtD) that encompasses the region after the Orange domain. Phosphorylation by CK2 relieves this CtD-mediated 'auto-inhibition'. M8* bypasses this cis-regulation because it lacks the CtD that would normally block the Orange domain. However, it requires N^{spl} to elicit a reduced eye phenotype because it cannot recruit the obligatory co-repressor Gro. It has been shown that N^{spl} renders R8's to become hypersensitive to inhibitory Notch signaling (Baker et al., 1996). It is under this sensitized condition that M8* becomes a potent repressor of Ato without its ability to bind Gro. In contrast, M8SD is a potent repressor in N^+ background because it still retains the ability to recruit Gro.

2.4. Study aims

The similar reduced eye phenotypes and interaction strength of M8SD or M8* to Ato suggest that the post-translational modification, i.e. phosphorylation, of the CtD regulates repression by M8. The following genetic studies were undertaken in order to understand the mechanistic basis of the phosphorylation and its effects on auto-inhibition.

I have analyzed the function of the CtD in the regulation of M8 activity by employing two variants of the CtD fragment that encompass only the C-terminal 56 amino acids from either wild-type M8 or M8SD. These are M8-CtD and M8SD-CtD, respectively. M8-CtD contains only the sequence after the Orange domain of wild-type M8. M8SD-CtD contains the sequence after the Orange domain of the phosphomimetic M8SD. Genetic analysis of transgenic flies expressing these two variants suggests that M8-CtD interacts and restrains neural repression by M8.

3. Materials and Methods

3.1. Plasmids and transgenic fly strains

Construction and germ-line transformation of UAS-m8-CtD and UAS-m8SD-CtD were previously carried out in our laboratory (Karandikar and Bidwai, unpublished) (Figure 2-2A). Transgenic flies were maintained on standard Yeast-Glucose media at 24 °C.

3.2. The Gal4-UAS system

The Gal4-UAS system was used to drive the misexpression of transgenes in a temporal- and spatial-specific manner (Brand and Perrimon, 1993; Duffy, 2002) (Figure 2-2). Expression of transgenes is under control of Upstream Activation Sequence (UAS) promoter which is activated by a yeast transcription activator Gal4. Mating between flies harboring enhancer-trapped Gal4 driver and transgenic flies generates progenies that contain both the UAS-transgene and Gal4 driver to allow for transgene expression in Gal4-dependent manner.

3.3. Flies and phenotypic analysis

To minimize variability of the phenotype, all crosses were conducted at 24 °C with 12hr day/night cycle, unless otherwise stated. In all cases, two or more independent insertions of each transgene were employed for studies. The Gal4 drivers used in the over-expression studies were gifts from Yuh Nung Jan (*109-68Gal4* and *scaGal4*), and Janice Fischer (*h^{H10}Gal4*). *N^{sp1}/Y* flies were kindly provided to us by Anette Preiss. Light microscopy images of fly eyes were used for average ommatidial count as a quantitative measurement to assess the effects of transgene expressions on eye development. Bristle phenotypes were analyzed by quantifying the percentage of transgene-expressing progenies with concerned defects, and by counting the number of macrochaetes present either on scutellum or notum depending on Gal4 driver employed. For scanning electron microscopy (SEM) analysis of the eyes, the whole flies were dehydrated by going through a

Figure 2-2: The UAS-Gal4 system and expression domains of Gal4 drivers

(A) The Gal4-UAS system allows the misexpression of transgenes in a temporal- and spatial-specific manner. Transcription of transgenes is under control of Upstream Activation Sequence (UAS) promoter which is activated by a yeast transcription activator Gal4. Mating between flies harboring enhancer-trapped Gal4 driver and transgenic flies generates progenies that contain both the *UAS-transgene* and Gal4 driver to allow for transgene expression in a Gal4-dependent manner. (B) Expression domains of Gal4 drivers used for over-expression studies of the CtD fragments in relation to the MF.

gradient of ethanol (25%, 50%, 75% and 100%) followed by immersion in Hexamethyldisilizane. Completely dehydrated flies were mounted on EM stubs with carbon tape (Ted Pella) and sputter coated with gold. They were examined using a JEOL-6400 scanning electron microscope at 20kV. The images were processed using the Adobe Photoshop and Adobe Illustrator.

3.4. Immunocytochemistry

Dissection of imaginal eye discs from late third instar larvae was conducted as previously described with minor modifications (Kavler et al., 1999). The dissected discs were fixed in 4% paraformaldehyde in 1X phosphate buffered saline (PBS) for 30 min at 4 °C. They were washed four times in PBS containing 0.1% Triton X (PBS-TX), each 10 min with shaking at room temperature. They were incubated for 14~16 h in PBS-TX containing 3% normal goat serum (NGS) and primary antibodies. Rabbit anti-Ato (gift from Yuh Nung Jan) and guinea pig anti-Sens (gift from Hugo Bellen) were each added at 1:500 dilutions. The discs were again washed four times in PBS-TX, each 10 min with shaking at room temperature. They were incubated for 3 hours at room temperature with shaking in PBS-TX solution which contains goat-anti rabbit-IgG coupled to Alexa Fluor 594 (dilution of 1:800) which fluoresces to give red color, and goat anti-guinea pig-IgG coupled to Alexa Fluor 488 (dilution of 1:800) which fluoresces to give green color. After four times wash with PBS-TX, the discs were mounted in Vectashield. Images were taken with an Olympus FluoView (FV1000) under an Olympus XL confocal microscope, and processed in Adobe Photoshop and Illustrator.

4. Results and Discussions

4.1. M8-CtD and M8SD-CtD elicits no aberrant eye phenotype

I have employed the Gal4-UAS system (Brand and Perrimon, 1993) to analyze the proposed role of the CtD as an auto-inhibition motif. I have misexpressed *UAS-m8-CtD* and *UAS-m8SD-CtD* by *scaGal4*, which drives expression in neural precursors in embryo and proneural clusters (PNC) within the morphogenetic furrow (MF) during R8/SOP specification (Hinz et al., 1994; Mlodzik et al., 1990; Nakao and Campos-Ortega, 1996). *scaGal4* was chosen based on the previous studies which showed that expression of M8SD elicits a severely reduced eye phenotype with this driver (Karandikar et al., 2004), akin to that elicited by *N^{sp1}/Y; E(sp1)^D/+* flies (Nagel and Preiss, 1999). M8SD is a phosphomimetic form of M8, which has been shown to be phosphorylated in vivo by protein kinase CK2 to become an active neuronal repressor during R8 development (Karandikar et al., 2004). In line with the proposed CtD-mediated auto-inhibition model, it was expected that the ectopically expressed CtD fragment of M8 would suppress the repressor ability of activated endogenous M8, i.e. M8Ser-159^{PO4}, which has already been relieved from its intra auto-inhibition (Figure 2-3). This suppression would be mediated by physical interaction between the M8-CtD fragment and the unhindered Orange domain of M8Ser-159^{PO4}, which would block the ability of the Orange domain to interact with proneural activator such as Atonal/ASC. In other words, ectopically expressed M8-CtD would inhibit repression by M8 in 'trans'.

Initially, eyes of the flies expressing the CtD variants were compared to those of wild-type flies by light microscopy (Figure 2-4). No aberrant eye phenotype was detected upon expression of M8-CtD or M8SD-CtD. Both the size and the pattern of the eyes were wild-type, and comparable between M8-CtD and M8SD-CtD. This indifference between two variants might be contributed to the buffering capacity inherent to an organism itself, which could handle a certain level of stochastic noise during development. Increasing expression dosage of the CtD fragment may cause enough disturbances to the system to

A. CtD constructs

B. 'Trans' Inhibition by M8-CtD

C. M8SD-CtD does not interact with the Orange domain

Figure 2-3 CtD constructs and their proposed in vivo effects

(A) Schematics of the ectopically expressed CtD fragments. (B) Over-expression of M8-CtD is thought to suppress the endogenous M8 activity 'in trans' by occluding the Orange domain of the phosphorylated M8 (M8^{PO4}) and thus inhibiting the interaction with proneural activators such as Ato/ASC. (C) M8SD-CtD is expected not to suppress the endogenous M8 activity because it would not be able to interact and occlude the Orange domain due to the phosphomimetic substitution (Ser159Asp).

observe the effect of the CtD fragment during R8 specification, if any.

4.2. M8-CtD and M8SD-CtD elicits ectopic bristles

Both M8-CtD and M8SD-CtD induced development of ectopic macrochaetes when expressed by *scaGal4* without significant difference between them (Figure 2-4). They also exhibited ectopic bristles only on scutellar region of the thorax. The % flies with ectopic bristle increased significantly from $2.4 \pm 2.8\%$ (WT) to $42.0 \pm 5.3\%$ in the case of M8-CtD, and $34.9 \pm 7.8\%$ in the case of M8SD-CtD (Student t's Test p-value > 0.0001 in both cases). When the average number of notum macrochaetes was quantified, flies expressing M8-CtD had 8.6, while those of M8SD-CtD had 8.4 bristles.

Ectopic bristles are an indication of impaired Notch function, possibly occurring at different stages of the SOP morphogenesis. It may imply loss of Notch signaling during the SOP refinement from a PNC (Hartenstein and Posakony, 1990), or during the division of pIIa which gives rise to shaft and socket. In either case, ectopic bristle phenotype induced by M8-CtD suggests that the endogenous M8 was not able to function normally to inhibit the activity of proneural activators ASC. If it is assumed that the expressed fragment is biochemically stable, as indicated by its ability to be phosphorylated by holoenzyme CK2 in vitro (Karandikar, U., and Bidwai, A. P., unpublished data), this phenotype may be mediated by the expressed CtD fragment that sequesters endogenous M8 from interacting with ASC or by sequestering CK2 from activating endogenous M8. This explanation of M8-CtD phenotype is in agreement with the auto-inhibition model discussed above. However, the effect of M8SD-CtD is surprising because it was expected that the phosphorylation relieves 'auto-inhibition' by the CtD.

4.3. Over-expression of CtD variants suppresses retinal defects of N^{spl}/Y

I next decided to employ a sensitized background, which may allow for assessment

Figure 2-4: Ectopic expression of the CtD fragments by *scaGal4*

Ectopic expression of M8-CtD or M8SD-CtD by *scaGal4* does not elicit eye phenotype, but both display ectopic macrochaetes (red stars). Presence of eight major notum macrochaetes (yellow circles in WT) was counted to quantify the average number of bristles on thorax, and the % flies affected with bristle defects were calculated.

of phenotypic differences between M8-CtD and M8SD-CtD. For these studies, I chose a recessive allele of Notch, N^{spl} . N^{spl}/Y flies display a reduced and rough eye phenotype, as well as split, missing and ectopic bristles (Cagan and Ready, 1989; Lindsley and Grell, 1967). These effects reflect sensitivity of the R8/SOP to inhibitory Notch signaling. In the wild-type, the R8/SOP's become refractory to Notch signaling, but this ability is impaired in N^{spl}/Y flies. This mutant background has been used previously by others to study $E(spl)$ functions. For example, $E(spl)^D$ shows no phenotype by itself, but elicits a severely reduced eye phenotype when expressed in N^{spl}/Y flies (Nagel and Preiss, 1999).

I have initially expressed M8-CtD and M8SD-CtD in N^{spl}/Y flies using *109-68Gal4*. This driver confines transgene expressions to PNC's that give rise to R8/SOP's (Doherty et al., 1997; Frise et al., 1996). Neither M8-CtD or M8SD-CtD altered the aberrant bristle phenotypes associated with the N^{spl} allele (data not shown). However, both M8-CtD and M8SD-CtD suppressed the reduced, rough eye phenotype of N^{spl}/Y flies (Figure 2-5). SEM analysis of the fly eyes expressing either variant showed substantial restoration of retinal patterning and size when compared to control flies (N^{spl}/Y ; *109-68Gal4/CyO*). I have attempted to quantify the restoration by counting the ommatidia of at least 40 flies from each genotype. While control flies had the average number of 388 ± 43 ommatidia per eye, flies expressing M8-CtD had 457 ± 52 , and those of M8SD-CtD had 481 ± 42 (both with p-value < 0.0001).

The CtD fragments were able to exert restraint on endogenous M8 during R8 specification, but not during SOP development when the expression was activated by *109-68Gal4*. Ectopic expression of M8 by *scaGal4* has been reported to suppress macrochaetes and macrochaetes, which implicates the role of M8 during bristle development (Giebel and Campos-ortega, 1997; Karandikar et al., 2004). Also, expression of CtD fragments by *scaGal4* in wild-type flies elicited bristle defects. The possible

Figure 2-5: Eye phenotypes of the CtD-variants with 109-68Gal4

N^{sp}/Y flies expressing different variants of the CtD by *109-68Gal4* which confines expression in proneural clusters. Control flies (*N^{sp}/Y; 109-69Gal4/CyO*) show reduced and rough eye defects associated with the *N^{sp}* allele. The size and the pattern of eyes expressing either M8-CtD or M8SD-CtD are substantially restored compared to the control flies. This restoration is significant (p-value < 0.0001) in both cases, which have been statistically evaluated by comparing the ommatidial number of 40 flies from each genotype.

explanation for this different effect of the CtD fragments on eye and bristle development in N^{spl}/Y flies could be that the activation by *109-68Gal4* may be too late or weak in the SOP development to allow for ample accumulation of CtD fragments to exert their effects. Earlier expression of the CtD fragment before R8/SOP specification might allow for enough build up of M8-CtD to inhibit endogenous M8.

The prior crosses were conducted at 24°C to minimize the variability of phenotypes between insertion lines. To assess whether increase in the expression level of transgenes may impact bristle development, I have conducted a parallel over-expression of M8-CtD and M8SD-CtD by *109-68Gal4* at 29°C. Due to the nature of Gal4 driver being a yeast transcription activator, higher expression of Gal4, thus stronger expression of transgene, is detected at 29C, which is the optimal temperature for yeast growth. This effect of temperature on transgene expression level has been reported in numerous studies and employed to increase the expression of transgene in transgenic lines without having to increase the dosage per se.

4.4. Over-expression of CtD variants by $h^{H10}Gal4$ suppresses both bristle and eye defects of N^{spl}/Y

I employed the driver, $h^{H10}Gal4$, to assess whether the CtD variants would affect both bristle and eye development of N^{spl}/Y flies. $h^{H10}Gal4$ driver was employed because it is known to activate the expression of transgenes prior to the MF (Ellis et al., 1994), and this earlier activation, in contrast to that of *109-68Gal4*, might allow for ample accumulation of transgenes in the PNC's before the SOP selection occurs. I computed the % flies with the aberrant bristle phenotype of the N^{spl} allele upon transgene expressions, which includes ectopic, split or missing bristles, to assess the effects of the CtD fragments on bristle development of N^{spl}/Y flies (Figure 2-6). Over-expression of M8-CtD or M8SD-CtD

Figure 2-6: Bristle phenotypes of the CtD variants with $h^{H10}Gal4$

Bristle defects associated with the N^{spl} allele (ctrl flies) includes ectopic (red star), split (yellow star) or missing bristles. These bristle defects are significantly suppressed by over-expression of M8-CtD or M8SD-CtD by $h^{H10}Gal4$, which activates transgene expression prior to the MF.

substantially suppressed the bristle abnormalities associated with the N^{spl} allele. Only $66.4 \pm 5.8\%$ of the flies expressing M8-CtD displayed bristle defects as compared to $92.2 \pm 0.9\%$ of control flies ($N^{spl}/Y; +/+; h^{H10}Gal4/+$) with the bristle abnormalities. $70.0 \pm 7.2\%$ of M8SD-CtD expressing flies exhibited the bristle defects.

The retinal size and pattern of the flies expressing either M8-CtD or M8SD-CtD were significantly restored compared to control flies which showed typical N^{spl} eye and bristle abnormalities (Figure 2-7). Although similar effects were also observed with 109-68Gal4, expressions by $h^{H10}Gal4$ seem have greater restoration effect in terms of retinal size and pattern. Also, higher magnification (1000x) of SEM eye analysis reveals that fewer ectopic IOB's are observed with either M8-CtD or M8SD-CtD than control eyes.

4.5. M8SD-CtD and M8SD-CtD substantially restores Ato and Sens in the MF

I have sought to assess how M8-CtD and M8SD-CtD elicit the suppression of N^{spl}/Y phenotype at the molecular level by observing Ato and Senseless (Sens) expression patterns. Ato and Sens are molecular markers for R8 specification in the developing eye discs of third instar larvae. Imaginal discs of control flies displayed low Ato expression prior to the MF which suggests that the cells were not gaining neural potency (through accumulation of Ato) with high efficiency. This is likely to be non-autonomous effect of N^{spl} ((Li et al., 2003), rather than an aberrant proneural activity of Notch in the PNC's (Nagel et al., 1999). The lack of morphogenic signals like Hedgehog (Hh), Decapentaplegic (Dpp) or Scabrous (Sca) secreted by the differentiating cells located posterior to the MF engenders the initial Ato expression in cells anterior to the MF (Greenwood and Struhl, 1999; Lee et al., 1996), which causes fewer number of PNC formation. Refinement of R8 cells from the PNC's was also less. Within the MF, many empty spots were visible, with more spaces between each R8 cell. Sens expression was not uniform, and some R8 cells expressed

Figure 2-7: Eye phenotypes of the CtD-variants with $h^{H10}Gal4$

Control flies ($N^{spl}/Y; +/+; h^{H10}Gal4/+$) show a reduced and rough eye phenotype associated with the N^{spl} allele. The size and the pattern of the fly eyes expressing either M8-CtD or M8SD-CtD are substantially restored in comparison to control flies. The restoration is significant (p-value < 0.0001) in both cases, which have been evaluated by comparing the average ommatidial count of 40 flies from each genotype (n=19 for control).

Sens at lower level, while some failed to maintain the Sens expression. Low number and level of Sens-expressing cells indicate that that R8 precursors were not able to maintain the neuronal R8 fate (Li et al., 2003). This is likely due to the sensitivity of R8 cells in N^{spl}/Y to inhibitory Notch signaling from neighboring cells (Li et al., 2003).

Immunostaining with anti-Ato and anti-Sens revealed that the number and the pattern of specified R8's were considerably restored in the discs from the M8-CtD- and M8SD-CtD-expressing larvae than those of control flies (Figure 2-8). Expression of M8-CtD and M8SD-CtD substantially restored expression of Ato anterior to the MF, which suggests that increasing number of cells posterior to the MF is undergoing differentiation and secreting Hh, Dpp and Sca to initiate Ato expression in non-differentiating cells. The restoration of Ato expression led to the increase in number and structured formations of PNC's along the D-V axis of the eye disc. Increasing number of PNC's and specified R8 cells agreed with the increase of average ommatidial counts and restoration of eye pattern and size of the adult flies.

Closer comparison of Ato expression patterns between M8-CtD and M8SD-CtD shows varying persistence of Ato expression. R8 cells in the discs of M8-CtD seem to lose Ato expression soon after they are specified, and begin to express Sens to maintain its R8 fate and to undergo further differentiation. However, Ato expression persists to 3-4 columns of cells after R8 specification in the MF in M8SD-CtD expressing discs. The implication of enduring Ato expression in M8SD-CtD is uncertain, but this difference in Ato expression pattern between M8-CtD and M8SD-CtD may be an indication that two variants are suppressing N^{spl}/Y phenotypes through different mechanisms.

4.6. Analysis of phenotype elicited by M8SD-CtD requires more investigations

In the auto-inhibition model discussed, phosphorylation by CK2 acts as a

Figure 2-8: Immunostaining of eye discs expressing the CtD variants

The eye imaginal discs of third-instar larvae from each genotype have been stained for Atonal and Senseless expression. The MF moves from poster to anterior region of the imaginal disc as indicated by the white arrow. Suppression of *N^{spl}* phenotype by M8-CtD or M8SD-CtD is apparent at molecular level by the substantial restoration of Ato and Sens expression in the MF. Note that the Ato expression lasts longer into several columns of cells in M8SD-CtD. This difference in the persistence of Ato expression between M8-CtD and M8SD-CtD may be indicative of their different actions on *N^{spl}*.

conformational switch that relieves M8 from its CtD-mediated repression. If the phosphorylation is indeed necessary and sufficient to relieve the auto-inhibition, it is expected that ectopically expressed M8SD-CtD would not interact with endogenous M8, which means it should not show any effect on bristle or eye development. However, M8SD-CtD elicits ectopic bristles like M8-CtD, with similar level of penetrance and expressivity. In addition, M8SD-CtD suppresses N^{spl} -associated retinal and bristle defects like M8-CtD.

Similar effects of M8-CtD and M8SD-CtD may be caused by the different level of transgene expressions. If M8SD-CtD is at much higher level than M8-CtD, due to their different location of insertions on the chromosome, massive presence of ectopically expressed M8SD-CtD could mask the system from undergoing its normal development. However, comparable results were obtained with other independent insertions with varying levels of expression strength as determined by eye pigmentation assay (data not shown) (Ephrussi and Herold, 1944). Also, the phenotypes elicited by M8SD-CtD and M8-CtD in adult flies were both specific to the developmental contexts in which they were over-expressed.

Highly possible situation is that the CK2-phosphorylation is not sufficient to relieve the CtD-mediated auto-inhibition. N-terminal to the CK2 recognition motif is a second stretch of highly conserved sequence, which has been termed the 'SPASSGY' motif in our laboratory. This motif is found only in a subset of E(spl) members (M5, M7 and M8) which also contains the CK2 site. The SPASSGY site is also found in M8 homologs of other species, and the mammalian homolog Hes6. The SPASSGY site contains several conserved Ser/Tyr residues that are targets for phosphorylation. Thus, it is possible that the secondary phosphorylation event may be required subsequent to the CK2 phosphorylation to engender a complete displacement of the CtD from the Orange domain. If this is the case, then M8-CtD and M8SD-CtD would display the same affinity and exert the same effect

Figure 2-9: Potential secondary phosphorylation

The 'SPASSGY' motif contains several highly conserved Ser/Tyr residues that may be targets of secondary phosphorylation subsequent to the CK2 phosphorylation as part of post-translational regulation. In this scenario, over-expression of M8-CtD or M8SD-CtD would repress the endogenous M8 activity, because both fragments would have similar binding affinity toward the endogenous M8.

of repressing the endogenous M8 activity when over-expressed in vivo.

From the genetic studies, it is highly suggestive that the C-terminal domain has an auto-regulatory function, but the mechanistic basis of the regulation is still in speculation because the crystal structure of M8 is not available. The position of the C-terminal domain in regard to the rest of the protein, especially the Orange domain, is still speculative. In addition, although phosphorylation of M8-CtD and M8SD-CtD by CK2 has been tested in vitro (Karandikar and Bidwai, unpublished), it is unclear whether the same is true in vivo. The actual dynamic of the phosphorylation-induced CtD displacement is likely to be more complex than the model discussed previously. It is possible that the displacement may be so minute, as it is the case with Ras which only moves by 2Å to expose its active site, that it would be hard to dissect out the role of the C-terminal domain in 'auto-inhibition' by over-expressing the CtD fragments.

5. Conclusions

Overexpression of M8-CtD elicits ectopic bristles, and suppresses bristles and retinal defects of *N^{sp1}/Y* flies. This effect is thought to be mediated by the direct/indirect interaction between the CtD fragment and the endogenous M8, which restrains repression by M8 during Notch-dependent lateral inhibition in eye and bristle development. However, M8SD-CtD seems to be capable of producing the similar effects elicited by M8-CtD. Immunostaining with R8 specification markers reveal some level of difference in the Ato expression pattern of M8-CtD and M8SD-CtD, which might indicate that the effect of M8-CtD is not mediated by the same mechanism as that of M8SD-CtD. Also, secondary phosphorylation subsequent to the CK2 phosphorylation remains an open possibility as post-translational regulation of M8 to relieve the auto-inhibition.

Chapter 3

The regulation of E(spl)M5 activity by CK2 phosphorylation.

1. Abstract

There are seven bHLH repressors encoded by the *Enhancer of split Complex* (*E(spl)C*) which are thought to be functionally redundant because of structural similarity and the lack of individual loss-of function allele. In vitro studies indicate that protein kinase CK2 phosphorylates M5 and M7 in addition to M8. I have investigated the functional significance of the CK2-phosphorylation of M5. I have employed molecular and genetic approaches similar to those previously used to assess the in vivo roles of M8 phosphorylation. To our surprise, over-expression studies with M5, and its CK2-variants, M5SA and M5SD, do not simply phenocopy the results obtained with similar variants of M8. These studies suggest functional specialization among *E(spl)* members.

2. Introduction

Earlier molecular and genetic analysis of E(spl) proteins contend that all bHLH members of *E(spl)C* are functionally redundant in the Notch pathway, without specific tasks assigned to each members (Delidakis et al., 1991; Schrons et al., 1992). This premise stems mainly from the lack of individual loss-of function or point-mutation phenotypes (Delidakis et al., 1991; Preiss et al., 1988; Schrons et al., 1992; Ziemer et al., 1988), and apparent similarity in phenotypes elicited when individual E(spl) proteins are misexpressed (Nakao and Campos-Ortega, 1996). However, the functional redundancy model has been in dispute for many years because accumulating molecular and genetic data suggest that there are significant differences in expression pattern and efficacy of neural suppression by individual members. The functional specialization model of E(spl) proteins can better fit and explain the complexity and various contexts that the Notch signaling is involved during development. Notch signaling, in which E(spl) members are the most commonly employed transcriptional targets, is extensively involved at different contexts of development, sometimes bearing different consequences. Notch activation in some instances leads to transcription repression (e.g. expression of *achaete* and *scute* during neurogenesis (Ruiz-Gómez and Ghysen, 1993; Skeath et al., 1992)), while, in others, leads to transcription activation (e.g. *wingless*, *cut* and *vestigial* expression during wing margin development (Couso et al., 1995; de Celis et al., 1996b; Rulifson and Blair, 1995)). To account for this kind of variations in the consequences of Notch activation, it is reasonable to conjecture that E(spl) proteins are utilized distinctively in different instances of the Notch signaling.

2.1. Differences among *E(spl)* members

Expression patterns

Since the transcription of *E(spl)* bHLH genes are Notch-dependent (Bailey and

Posakony, 1995; Jennings et al., 1994), one would expect to find all seven members to display the identical expression pattern in response to Notch activation. Studies of early embryonic neurogenesis demonstrate that, except for *m3*, all six *E(spl)* genes have in fact indistinguishable expression patterns (Knust et al., 1987; Knust et al., 1992). However, the expression patterns of individual genes in the imaginal discs are distinct (de Celis et al., 1996a; Jennings et al., 1999), which suggests the presence of additional regulatory factors that regulate the transcription of *E(spl)* genes, in addition to Notch and Su(H) (Cooper et al., 2000; Nellesen et al., 1999). In the developing eye imaginal disk, *m8*, *m7*, *mA*, *mB*, and *mC* are all detected at some levels near the MF. While *m8* expression spans several columns of cells within the MF along the dorsal-ventral axis, expression of *mA*, *mB* and *mC* is detected within discrete clusters of cells immediately posterior to the MF, and also within clusters of cells that are undergoing secondary specification in more posterior regions (de Celis et al., 1996a). In the wing disk, *m8* and *m7* are expressed in the SOP's that later give rise to mechanosensory bristles on thorax (de Celis et al., 1996a; Hinz et al., 1994; Lecourtois and Schweisguth, 1995). *mB* and *mC* are expressed only in subset of SOP's, and the *mA* transcript is found along the wing blade where the future wing veins are being determined. No transcript of *m5* and marginal level of *m3* are detected in the wing imaginal disk. However, it should be noted that 'lack' of expression might reflect levels that are below the 'threshold' for detection.

Protein-protein interactions

It is known that *E(spl)* proteins interact with proneural activators such as ASC or Ato through their HLH domain (the 'Orange' domain) to mediate repression. Two-hybrid protein interaction assays in yeast demonstrate that each *E(spl)* protein has preferential interactions with different set of proneural activators (Alifragis et al., 1997). In their studies, *E(spl)* members were classified into subgroups depending on their binding preferences. For

example, M5 and M8 showed preferential binding to the ubiquitous bHLH E-protein Daughterless (Da), while M3 showed preferential binding to ASC and Ato. Both MA and M7 interacted with ASC, Ato and Da at equivalent level.

Misexpression studies

Although effect of misexpression depends in part on the types of Gal4 driver used, in most cases, the efficacy with which E(spl) proteins suppress neurogenesis appears to be strongest when they are ectopically expressed in their wild-type functional domains (Ligoxygakis et al., 1999). In general, misexpression of E(spl) proteins during bristle development elicits bristle suppression (Nakao and Campos-Ortega, 1996; Tata and Hartley, 1995). But there are variations in severity of bristle loss and type of bristles being affected depending on which E(spl) members were misexpressed. In some cases, ectopic expression of some E(spl) repressors did not elicit any phenotype, while the others were potent repressors. M8 and M7 are the most potent out of all seven members at affecting notum macrochaete, notum microchaete and wing margins bristles (Ligoxygakis et al., 1999). M5 is the least potent, displaying very mild bristle suppression only when two copies of the gene are ectopically expressed (Ligoxygakis et al., 1999).

Studies in the eye have also demonstrated a degree of distinctions among E(spl) members. For example, ectopic M8 potently suppress IOB development, but does not affect R8 specification (Bose et al., 2006; Karandikar et al., 2004; Nagel et al., 1999). Therefore, the adult eye displays the normal hexagonal phasing of the ommatidia. In contrast, ectopic MC is a potent repressor of both the IOB and the R8 fate through down-regulation of *ato* expression during R8 specification (Baker et al., 1996; Dokucu et al., 1996; Ligoxygakis et al., 1998). As a result, ectopic MC leads to a reduced eye phenotype. But over-expression of M5 (Kim and Bidwai, unpublished) or MA has no consequence on R8 specification (Ligoxygakis et al., 1998). Once again, these differences among E(spl) proteins may reflect

qualitative differences between E(spl) members, although the nature of these differences have remained unclear.

Evolutionary conservation

Although it is common in mammals, the existence of genes with 'redundant' functions is rare in *Drosophila*. Even the genes with high degree of structural homology such as engrailed and ase display distinct functions and phenotypes when individual genes are point-mutated (Domínguez and Campuzano, 1993; Simmonds et al., 1995). Moreover, organization of *E(spl)C* genes is highly conserved, both in number and order, between *D. melanogaster* and *D. hydei*, which have diverged ~60 million years ago. M8 homologues from two species share more sequence similarity to each other (~ 77%) than to other members of its own E(spl) family (Maier et al., 1993). Such evolutionary conservation highly suggests and supports the functional specialization of individual E(spl) proteins during Notch-dependent processes. It is possible that the absence of individual loss-of function phenotype is due to the subtlety of elicited phenotypes that cannot be distinguished by the current genetic and/or molecular means, or lack of appreciation for buffering capability of development to small changes in potencies of individual E(spl) members.

2.2. *E(spl)M5*

The involvement of E(spl)M5 in the Notch pathway during neurogenesis of imaginal discs is the least known compared to other E(spl) proteins. Transcription of *m5* is detected during early neuroectodermal development in embryo, but expression in imaginal discs is not well characterized, perhaps because *m5* transcript is the least stable of E(spl) family (Ligoxygakis et al., 1999). E(spl)M5 demonstrates more homology to M8 than any other members of E(spl) (Delidakis and Artavanis-Tsakonas, 1992; Maier et al., 1993). Early expression patterns of the two genes (*m5* and *m8*) in embryo are indistinguishable, although

their expression patterns in the imaginal discs are quite different (Jennings et al., 1994; Ligoxygakis et al., 1999). While *m8* mRNA is found in most sensory organ precursors, that for *m5* is not detected in the wing imaginal disc. Preferential interactions of these two E(spl) proteins with Da, but not with Ato or ASC, are also similar (Alifragis et al., 1997; Gigliani et al., 1996). Albeit weaker than the effects elicited by ectopic M8, persistent expression of M5 in the imaginal discs has been reported to hamper the development of notum macrochaetes and microchaetes, and wing margin bristles (Nakao and Campos-Ortega, 1996). This was an evidence had led, in part, to the notion that these are 'general' repression of neurogenesis. Also, defects in SOP differentiation are observed as double sockets when *m5* is over-expressed (Nakao and Campos-Ortega, 1996).

2.3. *E(spl)M5* and protein kinase CK2

M5 has been found to be phosphorylated by protein kinase CK2 along with two other E(spl) proteins, M8 and M7. The significance of CK2 phosphorylation of M8 has already been discussed in Chapter 2. This additional layer of regulation would allow for selective activation of a subset of E(spl) proteins and thus functional divisions among E(spl) proteins. Although explicit phosphorylation assay with CK2-variants of M5 is required to map the site of phosphorylation, M5 is thought to have one CK2 phosphorylation site at Ser-156 in accordance with sequence homology to M8 and the presence of only one CK2 recognition consensus (Trott et al., 2001b).

2.4. Study aims

In an effort to better understand E(spl)M5, I have analyzed the phenotypic effects caused by ectopic expression of M5 using the UAS-Gal4 system (Brand and Perrimon, 1993). This is a continuation of the Honors research I have conducted in our laboratory during last two years of my undergraduate years. Different enhancer-trapped Gal4 drivers

have been employed to express three constructs at different stages of neurogenesis. In parallel, I have also investigated the functional significance of the CK2 phosphorylation in the regulation of M5 activity. I have generated CK2-specific variants of M5 and transgenic fly lines harboring these variants. I have conducted preliminary studies using these transgenic flies in combination with different Gal4 drivers and genetic backgrounds. In line with the notion of functional specialization among E(spl) members, the effects of expressing CK2-variants of M5 are strikingly different from the phenotypes elicited by the corresponding variants of M8.

3. Methods and Materials

3.1. Bacterial and yeast strains

Escherichia coli strain DH5 α was the bacterial strain used for all plasmid constructions and molecular manipulations. Bacterial transformants were selected and maintained on Luria Broth (LB, 1% yeast extract, 2% peptone, 0.5% NaCl) supplemented with ampicillin (150 μ g/ml) at 37°C.

Saccharomyces cerevisiae EGY048 (MATa, *trp1*, *ura3*, *his3*, *leu2::p3LexA_{op}(X6)-LEU2*) (Zervos et al., 1993), harboring *pSH18-34 (LexAop-lacZ)* (Estojak et al., 1995) was the yeast host used for the two-hybrid interaction assay. Yeast transformants were selected on selective drop out media with dextrose as a carbon source (SDD) supplemented with essential amino acid nutrients at 29°C. At times, yeast cells were cultured in dextrose rich medium (YPD, 1% yeast extract, 2% peptone, 2% dextrose) or galactose rich medium (YPG, 1% yeast extract, 2% peptone, 2% galactose).

3.2. M5, M5SA, and M5SD constructs

The *m5* cDNA was PCR amplified from *LexA-m5* (gift of David Ish-Horowicz) using a set of primers, R1KozIFM5 (5'-ggGAATT**CAAC**atgGCACCACAGAGCAACAACAGC-3') and M5Xho1P1 (5'-ccCTCGAGttaCCAAGGGCGCCACATGG-3'), which introduced EcoR1 (underlined, GAATTC) and Xho1 restriction sites (underlined, CTCGAG) at the 5' and 3' end of the *m5* cDNA, respectively, and in framed Kozac sequence (bold, CAAC) between the start codon (italicized small case, atg) and EcoR1 site. The italicized, small case 'tta' encodes for stop codon in the reverse primer. The PCR product was subcloned into the EcoR1 and Xho1 sites of pBluescript KS(+) (Stratagene), yielding the plasmid *pBS(KS+)-m5*, which was completely sequenced using a custom primer M13-21 (5'-GTAAAACGACGGCCAGT-3').

CK2-variants, *m5SA* (which replaces Ser156 with Ala) and *m5SD* (which replaces Ser156 with Asp) were made from the plasmid *pBS(KS+)-m5* via the Quick-Change Site-Directed Mutagenesis (SDM) kit (Stratagene), which is a modified version of regular PCR that allows introduction of mutation into a plasmid during amplification. The two primers used for *m5SA* variant are m5SA-P1 (5'- CCTCGGGATACCAC**GCC**GATAACGAGGACTC - 3') and M5SA-P2 (5'- GAGTCCTCGTTATC**GGC**GTGGTATCCCGAGG -3'), and those for *m5SD* variant are m5SD-P1 (5'- CCTCGGGATACCAC**GAC**GATAACGAGGACTCTC -3') and M5SD-P2 (5'- GAGAGTCCTCGTTATC**GTC**GTGGTATCCCGAGG -3'). The bold, underlined bases correspond to the location of Ser-156, which is replaced with bases (with codon bias) encoding Ala in the case of *m5SA*, or Asp for *m5SD* in the above primers, respectively. The SDM products were treated with the enzyme DpnI to remove the non-mutated template plasmids, and transformed into *E. coli* DH5_. The final constructs of two variants, *pBS(KS+)-m5SA* and *pBS(KS+)-m5SD*, were completely sequenced using the custom primer M13-21.

3.3. *M5* construct*

The *m5** cDNA was constructed from the plasmid *pBS(KS+)-m5* by SDM using the primers *m5*-P1* (5'- GTCGAGTTCCAGCGCATG**TAG**CAGGCCGATCAGGTCCAG -3) and *m5*-P2* (5'- CTGGACCTGATCGGCCTG**CTA**CATGCGCTGGA^{ACTCGAC} -3') to generate *pBS(KS+)-m5**. The primers introduce a stop codon (TAG) right after the Orange domain so that the transcribed *m5** cDNA lacks all the sequences C-terminal to the Orange domain. This was designed based on the *E(spl)^D* sequence.

3.4. Constructs for two-hybrid assay and transgenic analysis

The cDNA's of *m5*, *m5SA*, *m5SD*, and *m5** contained in the plasmid vector *pBS(KS+)* were each subcloned into *EcoR1* and *Xho1* sites of the following plasmid vectors: *pJG4-5* for the LexA-based two-hybrid analysis, and *pUAST* which expresses proteins with Upstream Activation Sequence (UAS) at the 5' UTR for over-expression studies in transgenic flies.

3.5. Primers and Sequencing

All the primers used for construction and verification of plasmids were synthesized by Integrated DNA Technologies Inc. (IDT). Primer sequences are described in Appendix 1. All the plasmids constructed for the studies are listed in Appendix 2. All PCR-based plasmids were confirmed by sequencing, which was performed by Molecular Genetics Instrumentation Facility (MGIF) at the University of Georgia.

3.6. LexA based two hybrid interaction assay

Explicit interactions of DmCK2 α and Groucho with M5 and its CK2-variants, M5SA and M5SD, were measured in the LexA-based two-hybrid assay referred to as the 'Brent' system because it was developed in the laboratory of Roger Brent (Gyuris et al., 1993). DmCK2 α and Groucho were contained in the plasmid vector LexA, which expresses the

protein as a fusion to the DNA binding domain of bacterial repressor LexA (LexA-DB). M5 and its CK2-variants, M5SA and M5SD, were contained in pJG4-5, which expresses the proteins as fusions to the activation domain of protein B42 (B42-AD). The yeast host EGY048 has a single chromosomally integrated *LEU2* gene under control of LexA operators, and the high-copy plasmid *pSH18-34* has *E. coli*-derived *LacZ* gene which is also under control of LexA operators. Two reporter genes, *LEU2* and *LacZ*, are induced when the interaction of DB- and AD-fusion proteins brings the DB-AD complex to LexA operators. In addition, expression of AD-fusion protein is only induced in the presence of galactose as a sole carbon source in media.

DB-DmCK2 α plasmid and AD-fusion plasmids of *m5*, *m5SA* and *m5SD* were transformed alone and in binary combinations of DB-plasmid and AD-plasmid into EGY048 containing *pSH18-34*. Transformants were selected on appropriate selective media. β -galactosidase (*LacZ*) activity was measured using ortho-nitrophenyl- β -galactoside (ONPG) as a substrate in a liquid assay to estimate the level of interaction between DB- and AD-fusion proteins (Miller, 1972). At least three independent transformants, each in triplicates, were tested for ability to induce *LacZ* gene. Transformants were grown overnight in selective media with galactose as a carbon source (SDG). These cells were subsequently cultured in YPG until they reached growth of mid-log phase, and optical density at 600 nm (OD_{600}) was measured. Cells were harvested and washed twice in 1X Z-buffer (60 mM Na_2HPO_4 , 10 mM KCl, 1 mM $MgSO_4$). 50mM β -mercaptoethanol in 1X Z-buffer was added, and cells were permeabilized in liquid nitrogen. Subsequently, ONPG in 1X Z-buffer was added, and cells were incubated at 30C for 15~30 min before stopping the *LacZ* reaction by adding 1M Na_2CO_3 . ONPG is hydrolyzed by *LacZ* to give a yellow color which is measurable at wavelength 420nm (OD_{420}). *LacZ* activity was measured in Miller units (Miller, 1972), which is calculated based on the formula $1000 \times OD_{420} / (T \times V \times OD_{600})$, where

T is minutes, and V is the concentration factor of the assay (normally 5).

3.7. Germ-line transformations and transgenic strains

Germ-line transformations of *UAS-m5SA*, *UAS-m5SD*, and *UAS-m5** were performed as described (Rubin, 1983) by Best Gene Inc. Balanced stocks of at least 5 independent insertions were established for each transgene, and mapped for insertion site on chromosome as previously described (Karandikar et al., 2004). Two independent transgenic lines of flies harboring *UAS-m5* were kindly provided to us by Yuh Nun Jan. Descriptions of all the transgenic lines are included in Appendix 3.

3.8. Flies and phenotypic analysis

Flies were maintained and crossed at 24°C on standard Yeast Glucose media. Over-expression of M5, M5SA, M5SD and M5* was achieved via the Gal4-UAS system (Brand and Perrimon, 1993). Analyses of eye and bristle phenotypes were carried out as described in Chapter 2 Methods and Materials.

4. Results and Discussions

4.1. Two-hybrid interactions of M5 variants to CK2 α and Groucho

CK2-variants of M5, M5SA and M5SD, have been designed according to the earlier studies with M8 and its CK2-variants and the conservation of CK2 recognition motif in M5, M7, and M8 (Figure 3-1A). Based on the sequence conservation to and analysis of M8, Ser-156 is the most likely phospho-acceptor in M5, which was substituted with Ala in the case of M5SA to mimic non-phosphorylatable state, and with Asp in M5SD to recapitulate the phosphorylated state. I have tested the interaction of M5 and its CK2-variants with DmCK2 α and Groucho using the LexA-based two-hybrid assay (Figure 3-1B). In this assay,

Figure 3-1: M5 variants and their interactions to CK2 and Groucho

(A) Schematics of M5, its CK2-variants, M5SA and M5SD, and M5*. The CK2 recognition sequence (in a box) is conserved in M5, M7 and M8. CK2 phosphorylates M5 at Ser-156, which is substituted with Ala in M5SA, and with Asp in M5SD, respectively. M5* is missing the sequence C-terminal to the Orange domain, as is the case in M8*. (B) Results from the LexA-based two-hybrid assay testing interaction of M5 and its CK2 variants (as fusions to B42-AD) with DmCK2 α and Groucho (as fusions to LexA-DB). M5 and M5SA show comparable interaction with CK2 α , while M5SD shows no interaction. Interaction of M5SD with Gro is twice as strong as that of M5 or M5SA. Representative data out of three independent experiments are shown. Each experiment was conducted in triplicates, from which standard deviation was calculated from the means. LacZ activities of DB- or AD-fusion alone clones are not shown as they were close to baseline.

DmCK2 α was fused to LexA-DNA binding domain (DB), while M5 and its CK2-variants were fused to the activation domain (AD) of protein B42. Undetectable or minimal level of LacZ activity was observed in the presence of DB- or AD-fusion protein alone (data not shown). Interaction of CK2 α with M5 or M5SA induced *LacZ* gene at a comparable level, indicating that CK2 α displays similar affinity towards either variant. On the other hand, a minimal level of LacZ activity, similar to that induced by DB or AD-fusion protein alone, was detected in a combination of CK2 α and M5SD. This suggests that CK2 α is not able to bind M5SD. In line with the presence of only one CK2 recognition motif in M5, this result supports that only one residue (Ser-156) is phosphorylated by CK2 in M5. This finding also suggests that the CK2-M5 complex might dissociate upon phosphorylation because CK2 α was no longer able to interact with the phosphorylated M5, i.e. M5SD. Phosphorylation assay of M5, M5SA and M5SD with CK2 would provide direct support for a single phosphorylation event by CK2.

M5 and M5SA also showed similar level of interaction with Gro, while M5SD interacted with Gro with an affinity that is twice as strong as that of M5 or M5SA. Gro has been shown to interact at high affinity with all the members of E(spl) proteins (Alifragis et al., 1997). The potential implications of increased interaction of M5SD with Gro are unclear at this stage. One possibility is that M5SD might be more stable, as compared to M5 or M5SA in vitro.

4.2. Over-expression of M5 elicits bristle loss

To assess whether phosphorylation by CK2 has any functional significance on in vivo activity of M5, I have over-expressed M5 and its CK2-variants by employing *scaGal4*, *c5Gal4*, *daGal4* and *pnrGal4* drivers. In line with the previous gain-of-function studies of M5, the most prominent phenotype induced by the expression of M5 was bristle loss, which

Figure 3- 2: Bristle phenotypes elicited by M5 and its CK2-variants

When M5 and its CK2 variants (M5SA and M5SD) are over-expressed by various Gal4 drivers, the general trend of elicited phenotypes is loss of bristles on thorax. Also, socket loss and thin bristle phenotype are observed. Loss of notum microchaetes is also detected when the expression is driven by *scaGal4* and *pnrGal4*. Ctrl indicates flies carrying only the Gal4 driver of the interest. (Ectopic bristle=red arrowhead, short bristle=purple arrow, socket only=blue circle)

included both the macrochaetes and microchaetes on the notum. (Ligoxygakis et al., 1999; Nakao and Campos-Ortega, 1996; Tata and Hartley, 1995) (Figure 3-2). Although the four Gal4 drivers have somewhat overlapping expression domains, different degrees of severity and types of bristle abnormalities were elicited when different drivers were used to activate transgene expression. The rank order of expression strength of these activators based on the severity of elicited bristle loss was: *scaGal4* > *daGal4* > *pnrGal4* > *c5Gal4*. Table 3-1 summarizes different phenotypes caused by each construct with focus on bristle loss. I have also noted other bristle abnormalities such as ectopic, short, or split bristle phenotypes. Penetrance of bristle defects were calculated by counting the number of affected flies, and expressivity of bristle loss phenotype was quantified by counting the occurrence of eight macrochaetes out of 22 that are normally present on wild-type notum. Figure 3-3 is a graphic representation to illustrate the penetrance of bristle loss phenotype elicited by M5, M5SA and M5SD for easy comparison between the efficacy of each transgene at mediating neural repression.

Neural repression by ectopic M5 was more prevalent than previously reported (Ligoxygakis et al., 1999) except when the expression was driven by *daGal4*. Expression of M5 by *scaGal4* and *pnrGal4* generated the most severe bristle loss phenotype (6.3~6.6 notum macrochaetes out of normal 8) with the highest level of penetrance (70~80% affected). With *daGal4*, only 19.9% flies experienced bristle loss with an average count of 7.8 ± 0.1 bristle per notum. Although both *c5Gal4* and *pnrGal4* drivers are expressed in wing imaginal disk which give rise to thorax region, different phenotypes were observed when they were used to activate the expression of *UAS-m5*. While bristle loss was observed when *pnrGal4* drove expression of M5, almost no bristle loss was observed (>0.1%) with *c5Gal4*. In fact, 14.2% of the progenies expressing M5 displayed an ectopic bristle phenotype, which may be indicative of abnormal SOP selection.

Table 3-2: Summary of bristle phenotypes elicited by M5 and its CK2-variants

scaGal4 >	M5	M5SA	M5SD
n	185	Lethal	60
% flies with bristle loss	70.3%		100%
Average notum bristle	6.6±0.6		3.2±0.5
Note	short bristle (11.9%) socket only (6.5%)		split bristle (5.3%) microchaete loss on thorax
daGal4 >	M5	M5SA	M5SD
n	261	semi-lethality (n=17)	197
% flies with bristle loss	19.9%	85.7%	93.7%
Average notum bristle	7.8±0.1	6.4±0.7	5.8±0.1
Note		short bristle (85.7%) ectopic bristle (14.3%)	
pnrGal4 >	M5	M5SA	M5SD
n	171	200	87
% flies with bristle loss	79.9%	100%	100%
Average notum bristle	6.3±0.7	1.9±0.4	3.8±0.5
Note	some microchaete loss on thorax	short bristle (36.5%) severe microchaete loss on thorax	short bristle (30%) some microchaete loss on thorax
c5Gal4 >	M5	M5SA	M5SD
n	344	348	74
% flies with bristle loss	>0.1 %	71.9%	32.4%
Average notum bristle	8.1±0.1	6.8±0.5	7.8±0.3
Note	Ectopic bristle (14.2%)	short bristle (91.8%) socket only (34.7%)	ectopic bristle (20.4%)

Figure 3-3: Expression of M5, M5SA and M5SD elicits bristle loss phenotype

The gain-of function phenotypes of M5, M5SA and M5SD were analyzed by driving the expression of transgenes with four different Gal4 drivers. The general trend is bristle loss on thorax region. M5SD causes the severest loss, followed by M5SA and M5.

4.3. Over-expression of M5SA and M5SD

M5SA and M5SD were more potent neural repressors than M5 (Figure 3-3). Expression of M5SA by *scaGal4* caused larval lethality, and semi-lethality/low viability when *daGal4* was employed. Their expression patterns may be the cause behind the lethality/semi-lethality because M5 is also required for neural precursor development during embryogenesis. Both drivers have stronger and earlier expression than *c5Gal4* or *pnrGal4*. *daGal4* causes ubiquitous expression throughout embryogenesis (Cronmiller and Cummings, 1993; Wodarz et al., 1995b), and *scaGal4* drives expression in the PNC's and the subsequent cell lineage in the MF (Mlodzik et al., 1990). Few escaped progenies (n=17) that expressed M5SA by *daGal4* had abnormal short bristles, and occasional ectopic bristles. M5SA expression by *pnrGal4* elicited strong repression of bristle development in all flies, with an average of 1.9 ± 0.4 bristles on notum. Also, a short bristle phenotype was observed with the frequency of 36.5%. Most of the microchaetes on thorax were also missing or short in length. When *c5Gal4* was used, almost all the flies had the short bristle phenotype, and 34.7% had a socket, but no shaft.

Although the general trend of bristle loss was the same as M5, expression of M5SA elicited other bristle defects such as short, ectopic or socket only phenotypes, which indicates aberrant Notch function, perhaps in the divisions following SOP specifications. The short bristle phenotype may be due to abnormal development of shaft cell. Socket only phenotype indicates Notch gain-of-function mutation after SOP selection, which results in all daughter cells from SOP gaining neuronal fates due to activated Notch signaling.

M5SD was not as potent as M5SA, but better than M5 at mediating neural suppression. Except for *c5Gal4*, expression of M5SD by three other Gal4 drivers led to bristle loss in >94% flies with the average number of notum macrochaetes ranging from 3.2 to 5.8 depending on the strength and domain of Gal4 driver used. When *pnrGal4* was

employed, short bristles were frequently (30%) observed. When the expression was activated by *c5Gal4*, M5SD induced ectopic bristles like M5, but with greater penetrance (20.4% affected in M5SD, compared to 14.2% in M5). Also, reduction of average number of bristles by M5SD, which is indicative of potency, was twice as great as that by M5.

4.4. Phosphorylation of M5 increases its neural suppression potency

All three variants, M5, M5SA and M5SD, were capable of repressing SOP development with varying degrees of potency. I have employed *g455.2Gal4* to further investigate the observed difference in the ability of M5 and its CK2-variants to suppress bristle development. The expression domain of *g455.2Gal4* is restricted in the triangular-shaped scutellum, which normally harbors 4 macrochaetes and no macrochaete. Because of the weaker activation strength of *g455.2Gal4* as compared to previously employed Gal4 drivers, M5 showed much lower penetrance of the bristle loss phenotype (8.7% affected).

Expression of M5SD, on the other hand, caused 59.7% of the flies to have missing bristles. Substitution of Ser-156 with Asp in M5SD increased the potency of M5 as neural repressor, which suggests that phosphorylation by CK2 augments M5 activity as a neural repressor in SOP development. Also, the results from the two-hybrid assay indicate that M5SD binds Gro at much higher affinity than M5. Gro-recruitment is required for all E(spl) members to exert its repression, which may possibly underlie the more efficient activity of M5SD as repressor.

M5SA was the strongest at suppressing bristle development, but it is unclear as to why M5SA acted more potent than either of M5 or M5SD when the CK2-phosphorylation site in M5SA was abolished. The possibility remains that these reflect difference in the expression levels or stability of each construct in vivo.

4.5. M5SD does not elicit a reduced eye phenotype observed with M8SD

G455.2Gal4 > UAS

Figure 3-4: Analysis of bristle loss by M5 and its CK2-variants

Severity of bristle loss elicited by M5 and its CK2-variants. Misexpression of transgenes by *G455.2Gal4* is confined to scutellum of the fly, which has four macrochaetes in wild type (WT). M5SA shows the strongest effect (94.7% flies show bristle loss), while M5 causes minimal bristle loss.

Over-expression of M5 and its CK2-variants suppressed sensory organ development on thorax, which was manifested as bristle loss. But over-expression of the same constructs did not elicit any eye phenotype when the expression was mediated by *scaGal4* which activates transgene expression in the MF, or by *daGal4* which has a ubiquitous expression pattern (Wodarz et al., 1995a). Inability of M5SD to elicit eye phenotype was surprising because it was expected that M5SD, but not M5, would have some effect on eye development. This expectation was based on the previous studies of wild-type M8 and its phosphorylated isoform, M8SD. Over-expression of wild-type M8 does not engender any eye phenotype, but M8SD causes a severely reduced eye (Karandikar et al., 2004), which led to the conclusion that phosphorylation is necessary for M8 to become an active repressor in eye development. Inability of M5, or M5SD, to elicit eye phenotype could be contributed to the fact that *m5* transcript is marginally detected in the eye imaginal disk in wild-type flies (de Celis et al., 1996a). Gain-of-function studies indicate that E(spl) proteins show the greatest potency as repressors when they are over-expressed in the domains where they are normally active in wild-type. Neither M5 or M5SD was able to reproduce the effects elicited by M8 or M8SD, respectively, during R8 specification, which suggests that E(spl) members may not play redundant functions as part of Notch signaling.

Another possible scenario is that M5 may be employed during later stages of eye development, such as specification of secondary photoreceptor cell fates, in which Notch is also known to be required. If secondary specifications were indeed affected by over-expression of M5 or its variants, it would require tangential sectioning of the adult eyes as these types of altered cell fates is not detectable in the morphology of adult eyes.

Bristle development may be more sensitive to the stochastic noise in the system than eye development, as previously observed in the CtD studies (Chapter 2). Also, it is possible that the lack of phenotypes may be due to the low expression level or instability of

Figure 3-5: Eye phenotypes elicited by M5 and its CK2-variants by *scaGal4*

Over-expression of M5, M5SA or M5SD has no effect on eye development when expression is driven by *scaGal4*, which is known to activate transgene expression in the PNC's within the MF.

proteins encoded by the transgenes in vivo. Thus, employing more sensitized background, or increasing dosage of transgenes may allow us to assess the effect, if any, of M5 variants on eye development.

4.6. M5 suppresses N^{spl} -specific phenotype

Rather than increasing the dosage of each of M5 variants, I decided to express the transgenes in combination with the N^{spl} allele in order to assess if M5 or its CK2-variants M5 have any effect on eye development. N^{spl} mutant background provides a sensitized background for Notch and E(spl) functions, and it was previously used to assess the CtD-dependent auto-inhibition of M8 in Chapter 2. I have used $h^{H10}Gal4$ to over-express the variants because its expression begins earlier time point in the region posterior to the MF. It was reasoned that earlier expression of transgenes might allow for sufficient amount of proteins to accumulate before R8 specification begins in the eye imaginal disk. In addition to M5SA and M5SD, I have also generated another construct, UAS-m5*, by inserting a stop codon at the end of Orange domain in the wild-type *m8* cDNA. This design was based on the $E(spl)^D$ allele, which enhances the effects of the N^{spl} allele on neurogenesis.

Expression of M5 suppressed retinal defects of N^{spl} by small margin (p-value = 0.0279), increasing the number of ommatidia from 317 ± 64 to 354 ± 48 (Figure 3-5). Although the effect of M5 expression in N^{spl}/Y flies is opposite to its known function in the Notch pathway, the small margin of suppression by M5 may not be as significant considering the large standard deviation value of the average ommatidial count. For example, when M5-expressing eyes were observed under SEM, the arrangement of ommatidia and size of the eye field was similar to that of control flies (Figure 3-6).

4.7. M5SA and M5SD enhance, while M5* has no effect on N^{spl} allele

Expression of M5SA or M5SD elicited a severely reduced eye with supernumerary

Figure 3-6: Eye phenotypes elicited by M5 variants in N^{spl}/Y flies

Light microscopic images of fly eyes expressing different variants of M5 in N^{spl}/Y flies with $h^{H10}Gal4$, which drives transgene expression anterior to the MF. Ectopic expression of M5SA and M5SD enhances a reduced, rough eye phenotype of N^{spl}/Y flies (control), while M5 and M5* generate no effect. Enhancement by M5SA and M5SD was determined significant ($p < 0.0001$) by comparing the average ommatidial count of flies expressing the transgenes to that of control flies.

IOB's (Figure 3-5 and 3-6). Empty regions between ommatidia were filled with clusters of ectopic IOB's. These spaces could be a result of irregular arrangement of ommatidia, or cells undergoing apoptosis. Supernumerary IOB's in clusters implies abrogated lateral inhibition during SOP refinement, in which case multiple SOP's are specified rather than one from each PNC. Also, eyes expressing M5SD had crater-like depression in their ommatidia, or 'blueberry' phenotype, which results from secondary photoreceptors or accessory cells (such as cone cells) undergoing apoptosis (Basler et al., 1990; Moffat et al., 1992). A few ommatidia in M5SA expressing flies also displayed a similar phenotype. Tangential sectioning of the adult eye would be required to find out the physiological cause behind this phenotype. This result implicates the possible role of M5 during secondary cell fate specification.

On the other hand, expression of M5* did not have any effect on N^{spl} phenotype. This phenotypic effect of M5* is contrary to that of M8*, which is the protein encoded by $E(spl)^D$ allele. M8* elicits a severely reduced eye with an average ommatidial count of 8 ~25 (Karandikar et al., 2004; Nagel et al., 1999). It is thought that M8* exerts dominant effect because of its ability to bind Ato with much higher affinity by the absence of the 'auto-inhibitory' C-terminal domain (as discussed in Chapter 2). If this 'auto-inhibition' model is also physiologically relevant in the case of M5, then the lack of C-terminal domain in M5* should have bestowed the similar dominant ability to M5*. This discrepancy between M5* and M8* supports the non-redundant roles of E(spl) proteins, and implicates the possibility that the role of CK2 phosphorylation on M5 may differ from that of M8. .

4.8. Role of CK2 phosphorylation on M5 activity during eye development

Expression of M5SD augmented the neural repression in N^{spl} males, while M5 had marginal effect, if any. This suggests that phosphorylation of M5 increases the repression ability of M5 during eye development. Higher activity of M5 repressor, as would be the

$N^{spl}/Y; h^{H10}Gal4 > UAS$

Figure 3-7: Eye phenotypes elicited by M5 variants in N^{spl}/Y flies

Adult eyes of control flies and those expressing M5 are virtually identical in terms of patterning and spacing of ommatidia. The number of ectopic IOB's at vertices of ommatidia is also similar. When flies expressing either M5SA or M5SD are compared to control flies ($N^{spl}/Y; +/+; h^{H10}Gal4/+$), supernumerary IOB's are observed with higher frequency throughout the eye fields, especially in the areas of missing ommatidial. Flies expressing M5SA have depressions in ommatidial, a phenotype referred to as 'blueberry' (red arrow), but this phenotype is more prevalent in flies expressing M5SD. Magnifications are as indicated

case with M5SD, in already sensitized background for N^{spl} and E(spl) protein functions, would lead to greater reduction of specified R8's, and possibly inducing apoptosis of proneural cells that could not acquire or maintain their proper cell fates. Also, it is possible that M5 may play a role during specification of secondary photoreceptors and/or accessory cells. The overall physiological consequence of the CK2 phosphorylation on M5 seems to parallel that of M8, which is to increase or activate repression activity of two E(spl) proteins. But the mechanistic basis of M5 phosphorylation may not involve the C-terminal domain as an 'auto-inhibitory' domain, as it has been proposed in the case of M8.

5. Conclusions

Studies so far indicate that M5 plays non-redundant role from M8, especially during eye development. M5SD does not phenocopy the effects elicited by M8SD, nor does M5* like M8*. In the case of bristle development, general trend of gain-of-function phenotype, i.e. bristle loss, is similar between M5 and M8. Both M5 and M8 display increase in their repression activity after CK2 phosphorylation, but the physical consequence of CK2 phosphorylation on their conformation may involve different mechanism. These result implicate different functional contributions of E(spl) proteins during Notch-mediated neurogenesis.

Chapter 4

Identification of interacting partners of M8SD-CtD using the yeast two-hybrid approach

1. Abstract

The studies described in Chapter 2 suggest that CK2 might not be the sole regulator of the auto-inhibited state of M8. It has been previously proposed that phosphorylation by CK2 engenders a conformational change which relieves M8 from its auto-repression. However, this model needs to be qualified because it is based upon the in vivo behavior of M8SD, a dominant phosphomimetic variant. The observations that CtD's of M8 or M8SD were both competent at eliciting dominant neural defects raised the possibility that auto-inhibition may involve motifs in addition to the CK2 site. This possibility is underscored by the observation that residues N-terminal to the CK2 phosphorylation site are highly conserved in a subset of E(spl) proteins, i.e. M7, M8, M5. This conserved sequence motif exhibits the inordinate conservation of Ser/Tyr residues. Moreover, these potential phospho-acceptors are also conserved in the Hes6, the mammalian homolog of *Drosophila* E(spl) proteins. In the case that CK2 phosphorylation were to act as a 'priming' event, the possibility exists that the observed trans-inhibitory properties of M8-CtD and M8SD-CtD might reflect the necessity for secondary phosphorylation. In order to identify any such factors that might lead to secondary phosphorylation or regulation, the M8SD-CtD fragment has been used in a two-hybrid screen to identify potential interacting partners of this sub-domain. These studies have led to the identification of a number of interacting partners.

2. Introduction

2.1. Secondary phosphorylation

The CK2 recognition motifs in E(spl)M5/7/8 are all preceded by a second highly conserved sequence (Figure 4-1). This conserved sequence is S-P-(A/V)-S-S-G-Y (heretofore, this sequence is referred to as the 'SPASSGY' motif). As stated above, this motif is also conserved in mammals. Considering that Ser or Tyr residues are targets for phosphorylation, the possibility arises that secondary modification of the SPASSGY motif could be a component of the auto-inhibition regulation. Alternatively, the CtD might recruit factors other than kinase whose binding to M8 influences repression.

Examples of secondary phosphorylation following a priming phosphorylation, i.e. sequential phosphorylation event, are abundant in biological world. For example, cAMP response element binding (CREB) protein is first phosphorylated by Protein Kinase A (PKA), which is then subsequently phosphorylated by Glycogen Synthase Kinase-3 (GSK-3) (Fiol et al., 1994). Like CK2, PKA belongs to the Ser/Thr kinase family, and is made up of two regulatory and two catalytic subunits. In addition, synergistic interaction between CK2 and GSK-3 has been reported, in which an initial phosphorylation by primary kinase, i.e. CK2, creates a recognition site for the secondary kinase, i.e. GSK-3. These interactions of CK2 and GSK-3 have been reported in a number of substrates such as Glycogen Synthase Phosphatase Inhibitor and the regulatory subunit of PKA (Fiol et al., 1987; Picton et al., 1982).

The secondary phosphorylation event, if any, is expected to be performed by a kinase other than CK2, because phosphorylation assays demonstrate that CK2 phosphorylates M8 only once at Ser-159. One of the potential secondary kinases is GSK-3. For example, the SPASSGY site contains a consensus recognition motif (SxxxS) for GSK-3.

M8	b	HLH	Orange	SPASSGY	CK2	Gro
<i>D. melanogaster</i> -M8:				S PASSGY H S DCDSPPP		
<i>D. simulans</i> -M8:				S PASSGY H S DCDSPAP		
<i>D. pseudoobscura</i> -M8:				S PASSGY H S DCESPAP		
<i>D. grimshawi</i> -M8:				S PASSGY H S DCESPAP		
<i>D. hydei</i> -M8:				S PASSGY H S DCESPMP		
<i>D. melanogaster</i> -M5:				S PASSGY H S DNEDSQS		
<i>D. melanogaster</i> -M7:				S PVSSGY A S DNESLLQ		
<i>human</i> -Hes6:				S PL SS -9- S DLEE		
<i>mouse</i> -Hes6:				S PI PS -9- S DLEE		

Figure 4-1: The SPASSGY motif

Sequence alignment of M5, M7, Hes6 (mammalian homolog of M8), and M8 from different *Drosophila* species show a second highly conserved sequence named the 'SPASSGY' motif (in yellow box), in addition to the CK2 recognition motif (in red box). Highly conserved Ser/Tyr residues (bolded) in the SPASSGY motif are potential targets for the secondary phosphorylation event that may occur subsequent to the CK2 phosphorylation. Schematic of M8 displaying other functional domains is not scaled to actual sequences.

Since collaborative action between CK2 and GSK-3 has already been reported in other contexts (see above), a similar type of sequential phosphorylation that may contribute to the regulation of M8 activity remains an open possibility.

2.2. Protein phosphatase 2 A (PP2A)

Recent studies conducted in our laboratory implicate Protein Phosphatase 2 A (PP2A) as a potential regulator of inhibitory Notch signaling (Kunttas-Tatli, Bose, and Bidwai, unpublished). Specifically, these studies suggest that the activity of PP2A appears to act opposite to that of CK2 during inhibitory Notch signaling. Thus, a PP2A gain-of-function mutant phenomimics the bristle and retinal defects of a CK2 loss-of-function mutant. It is therefore expected that proteins that interact with M8SD-CtD might include a phosphatase, or other novel proteins whose relevance to repression by M8 was previously unknown.

2.3. Study aims

The summary of potential interactions/regulations of M8 is shown in Figure 4-2. In order to determine proteins that interact with the CtD, a two-hybrid screen has been conducted. The M8SD-CtD tethered to the DNA binding domain of Gal4 was employed as bait to screen a 3-18 hour embryo cDNA library. The cDNA library, which expresses proteins as C-terminal fusions with the activation domain of Gal4, has previously been used to identify the interaction between CK2 α and E(spl)M7. The interaction of the bait and the target protein was detected by the induction of two reporter genes, *HIS3* and *LacZ*.

While the choice of M8SD-CtD as bait might seem counterintuitive, it was reasoned that a screen with a full-length M8SD would lead to the identification of proteins whose interactions with M8 are already known. To avoid, the 're-identification' of these well-known interacting proteins, the CtD of M8SD was used with the expectation that this fragment might identify novel partners. Previous biochemical analysis of the isolated M8SD-CtD indicates

Figure 4-2: Post-translational regulations of M8

In addition to the CK2 phosphorylation, potential post-translational regulations of M8 activity have been proposed. Highly conserved Ser/Tyr residues in the SPASSGY motif may be targets for phosphorylation by secondary kinases. Protein Phosphatase 2 A (PP2A) has been implicated as a potential regulator of M8 subsequent to the CK2 phosphorylation. Also, there is a possibility of other factors (X-factor) that might influence M8 activity.

that the fragment displays biochemical characteristics that are comparable to that of the full-length M8SD (Karandikar and Bidwai, unpublished). For example, M8-CtD displays robust interaction with CK2, whereas that of M8SD-CtD is negligible. In contrast, both fragments interact robustly with Gro. Thus, the CtD's appear to recapitulate at least the interactions with CK2 and Gro.

3. Methods and Materials

3.1. Bacterial and yeast strains

E. coli strain DH5 α was the bacterial strain used for the constructions and molecular manipulations of plasmids. Bacterial transformants were selected and maintained on Luria Broth (LB, 1% yeast extract, 2% peptone, 0.5% NaCl) supplemented with Ampicillin (150 μ g/ml) at 37°C.

S. cerevisiae HF7c (*MAT α* , *ura3-52*, *his3-200*, *ade2-101*, *lys2-801*, *trp1-901*, *leu2-3, 112*, *gal4-542*, *gal80-538*, *LYS2::GAL1_{UAS}-GAL1_{TATA}-HIS3*, *URA3::GAL4_{17mers(x3)}-CyC1_{TATA}-LacZ*) (Feilotter et al., 1994) was the yeast host used for the two-hybrid screen. Yeast transformants were selected and maintained at 29°C on selective drop out media with dextrose as a carbon source (SDD). 1 mM 3-aminotriazole (3-AT) was added when needed.

3.2. Construction of two-hybrid plasmids

The coding sequences of *m8-CtD* and *m8SD-CtD* including in frame Kozac sequence (CAAC) at the 5' UTR were excised out from previously constructed plasmids pBSII(SK+)-*m8-CtD* and pBSII(SK+)-*m8SD-CtD*, respectively, by restriction digest with EcoR1 and BamH1. Isolated cDNA's were each subcloned into the EcoR1 and BamH1 sites of the plasmid vector pGBT9 (Clontech), yielding the plasmids pGBT9-*m8-CtD* and pGBT9-

m8SD-CtD, respectively. The resulting plasmids express M8-CtD and M8SD-CtD as the C-terminal fusions to the DNA binding (DB) domain of *S. cerevisiae* transcriptional activator Gal4 (Gal4DB). Two plasmids were transformed into HF7c using lithium acetate (Guthrie and Fink, 1991). The transformants were isolated on selective dropout media (SDD) lacking Trp and Leu.

3.3. Gal4-based yeast two-hybrid screening

The Gal4-based two-hybrid screen was conducted as previously described with some modifications ((Trott et al., 2001b). The two-hybrid cDNA library was generated from 3-18 h *D. melanogaster* embryos (gift of S. J. Elledge, Baylor College of Medicine). The library was inserted in the Xho1 site of the plasmid pACT, which expressed cDNA's (target) as C-terminal fusions to the activation domain of *S. cerevisiae* transcriptional activator GAL4 (GAL4AD) (Durfee et al., 1993)). The plasmid pGBT9-m8SD-CtD was used as bait to screen the library. The interaction between the bait and the target protein was detected by the induction of reporter genes, *HIS3* and *LacZ* (Chien et al., 1991). HF7c which already contained the bait plasmid was transformed with the cDNA library. A total of 3.0×10^7 transformants was plated on SDD lacking Trp, Leu and His and supplemented with 1 mM 3-AT. Trp and Leu were also missing in selective media in order to allow growth of only the transformants containing both the bait (marked with TRP1) and target plasmids (marked with LEU1). 1 mM 3-AT was also added to suppress residual *HIS3* expression. 3-AT is a chemical inhibitor of Imidazole glycerol phosphate dehydratase, which is an enzyme required for in vivo production of His (Kishore and Shah, 1988). The optimal concentration of 3-AT to suppress the basal transcription of *HIS3* was determined by testing concentrations ranging from 0.1 mM to 10 mM with proper positive and negative controls. Of 1119 mid-to-large sized clones growing on selective media, 1080 clones were tested positive for *HIS3* and *LacZ* induction. *LacZ* induction was tested using colony-lift filter assay

(Breedon and Nasmyth, 1985) which measures blue color generated by hydrolytic activity of β -galactosidase with X-gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) as a substrate. First 100 His⁺/LacZ⁺ colonies were streaked out to obtain pure clones, which were again checked for ability to induce *LacZ*. 40 pure clones were induced to drop the bait plasmid by sub-culturing in SDD lacking only Leu for 10 days. Drop-out clones were confirmed by replica-plating on SDD lacking Leu, and SDD lacking Leu and Trp. Pure clones that grew on SDD lacking Leu were selected for further analysis. The cDNA plasmids from 14 drop-out clones were isolated and transformed into *E. coli* DH5 α . PCR was performed with the primers THS-AD1 (5'-ATACCACTACAATGGATGATG-3') and THS-Gro (5'- CGC GGT GAG TGG GAT TCC ATT TCA TTT GCA -3') to eliminate any Gro-coding cDNA. None of the clones produced the PCR product, and thus were sequenced using the primer THS-AD1. BLAST analysis was performed to determine the identity of the encoded proteins.

3.4. Primers and Sequencing

Primers used for verification of cDNA plasmids were synthesized by Integrated DNA Technologies Inc. (IDT). Primer sequences are described in Appendix 1. All the plasmids constructed for the studies are listed in Appendix 2. Sequencing of cDNA plasmids was performed by Molecular Genetics Instrumentation Facility (MGIF) at the University of Georgia. Chromatograms are included in Appendix 3.

4. Results and Discussions

4.1. Preliminary characterization of the CtD fragments

In the two-hybrid screen, protein-protein interactions led to transcription of reporter

genes, *HIS3* and *LacZ*. Transcription of the former gene complements the nutritional (His) auxotrophy of the yeast host HF7c, and thus enabled isolation of the clones on minimal medium lacking His. In addition, transcription of *LacZ* gene served as a counterscreen. It is important to note that success with the screen depended on the induction of *HIS3* only in response to protein-protein interaction. Any baseline (low level) *HIS3* induction allows for growth in HIS⁻ medium, a problem that has been encountered for a number of baits. This baseline transcription of *HIS3* reflects weak transcriptional activating properties of the bait-fusions.

For the reasons mentioned above, the yeast strain HF7c was transformed with Gal4DB-M8SD-CtD or Gal4DB-M8-CtD. These transformants were tested for their ability to induce baseline *HIS3* transcription. As shown in Figure 4-3, cells expressing M8-CtD alone could not grow on medium lacking His. In contrast, cells expressing M8SD-CtD alone were found to exhibit detectable growth on medium lacking His. This result suggests that the Asp substitution engenders weak trans-activating properties to the CtD. Thus, different concentrations of 3-AT were used to determine the minimal level of this inhibitor that would silence the baseline expression of *HIS3*. It was found that 1 mM 3-AT was sufficient to block growth on His⁻ medium. As added controls, both CtD fragments were tested for their ability to interact with CK2. It was found that cells coexpressing M8-CtD + CK2a grew on His⁻ medium (with or without 1 mM 3-AT). In contrast, M8SD-CtD displayed no growth on medium supplemented with 1 mM 3-AT. Thus, the screen was conducted in selective media with 1 mM 3-AT.

4.2. Screening a cDNA library with M8SD-CtD

The yeast strain HF7c harboring the bait plasmid pGBT9-m8SD-CtD was transformed with the Gal4-AD fusion cDNA library as described in *Methods and Materials*

Figure 4-3: Test for *HIS3* induction

The residual *HIS3* transcription was observed in clones containing only M8-CtD or M8SD-CtD. Varying concentrations (0 mM~10mM) of 3-AT, which inhibits *HIS3* production, were tested. It was found that 1 mM 3-AT was sufficient to silence the baseline expression. As added controls, both CtD fragments were tested for their ability to interact with CK2. It was found that cells coexpressing M8-CtD + CK2a grew on His- medium (with or without 1 mM 3-AT). In contrast, M8SD-CtD displayed no growth on medium supplemented with 1 mM 3-AT. Thus, the screen was conducted in selective media with 1 mM 3-AT.

(Figure 4-4). A total of 3×10^7 independent transformants were obtained. These transformants were tested for their ability to grow on Trp⁻Leu⁻His⁻ media containing 1 mM 3-AT. Sizes of the growing colonies varied from large (>5 mm) to small (<1 mm) in diameter. For arbitrary reasons, the threshold colony size was chosen to be ≥ 2 mm. Of the original pool of transformants, 1119 clones were selected for further analysis. These clones were individually analyzed for expression of *LacZ* using a qualitative filter-based assay (Figure 4-4C). Of the total, 39 clones did not express *LacZ* at levels that were detectable, and for this reason, they were discarded. The remaining 1080 clones were classified based upon the levels of *LacZ* induction. These clones were archived and a subset (30 clones) was selected for further analysis.

4.3. Isolation of drop-out clones

In order to determine whether *HIS3* and *LacZ* induction of the isolated clones required the bait, 30 clones were grown under non-selective conditions, and those clones that had dropped the bait plasmid were selected for on medium lacking only Trp. These clones thus contained the cDNA library plasmid by itself. If the cDNA encoded for a protein that allows for binding to the Gal4 promoter (upstream of *HIS3* and *LacZ*), these clones are expected to induce reporter expressions in a bait-independent manner. Of the 30 clones subjected to drop-out analysis, none elicited the expression of *LacZ*. These 30 clones were archived as bait-dependent interacting clones.

4.4. Sequencing of cDNA's from drop-out clones

The library plasmids of the 30 drop-out clones, which induced *LacZ* at varying levels, were recovered into *E. coli*. PCR analysis was used to eliminate any clones that encoded Gro, which was expected given that the CtD fragment contains a fully functional WRPW (Gro-binding) motif. Of the total, 14 clones were sequenced.

A. Transformation of cDNA's into bait-containing host

B. Screen procedure

C. *LacZ* Induction test

Figure 4-4: Schematics of the library screen procedures

(A) Interaction between the bait and the target cDNA leads to induction of *HIS3* and *LacZ* genes. (B) The two-hybrid screen procedures. (C) Filter-based *LacZ* induction assay. A grade of blue color is observed depending on the interaction strength of bait and target proteins. Clone containing only the bait-plasmid does not give blue color, while clone containing interacting bait and target generates blue color.

4.5. Identities of interacting cDNA's

The identities of the encoded proteins were determined by BLAST analysis against the *Drosophila* genome (see Table 4-1). As expected, three of them encode ribosomal proteins, which have also been identified in a number of two-hybrid screens with diverse baits (Hengen, 1997). The likelihood is high that the isolation of these ribosomal proteins reflects interactions with baits that are non-physiological. Similarly, five clones encode proteins that are either secreted or predicted to have functions in metabolism. Given that E(spl)M8 is a nuclear-localized transcriptional repressor (Delidakis et al., 1991), it is unlikely to ever be in the same compartment as secreted proteins. In addition, there is no evidence that E(spl)M8 is involved in cellular metabolism. For these reasons, these proteins are not discussed further.

Of interest are three clones, which encode nucleoplasmin, (muscle) LIM protein, and suppressor of white-apricot (SWAP). The known functions of these proteins in *Drosophila* or other organisms are discussed in the following section.

Nucleoplasmin

In *Drosophila*, nucleoplasmin is also referred to as nucleoplasmin-like protein (dNLP) (Ito et al., 1996b) or chromatin decondensation protein (CRP1) (Crevel et al., 1997). Nucleoplasmin is a nuclear chaperone, which belongs to a family of proteins that are referred to as nucleophosmin/nucleoplasmin (NPM). Collectively, these proteins are thought to function as nuclear chaperones in metazoan organisms (for reviews, see (Eirín-López et al., 2006; Frehlick et al., 2007)). Nuclear chaperones coordinate an ordered assembly of nucleo-protein complexes, such as histones or ribosomes. The NPM family also includes *Drosophila* NAP-1 (Ito et al., 1996a), *Xenopus* N1 and N2 (Kleinschmidt et al., 1986), NO38/nucleoplasmin (Schmidt-Zachmann et al., 1987), and nucleolin (Lapeyre et al., 1987)

as well as mammalian homologs such as NAP-1 (Ishimi et al., 1985), nucleophosmin/B23 (Chan et al., 1989) and CAF-I (Smith and Stillman, 1989).

Studies of nucleoplasmin homologs from mammals and amphibians have revealed a number of different biological roles played by the NPM proteins. Extensive studies of *Xenopus* nucleoplasmin demonstrated roles in remodeling (decondensation) of sperm chromatin after fertilization (Philpott and Leno, 1992; Philpott et al., 1991) and coordination of histone assembly onto DNA during nucleosome organization (Okuwaki et al., 2001). Mammalian homologs are reported to have nucleic acid binding property (Dumbar et al., 1989) and ribonuclease activity during pre-ribosomal RNA processing (Herrera et al., 1995). They have been implicated in stimulation of DNA polymerase α in synergistic interaction with retinoblastoma protein (Takemura et al., 1999), and centrosome duplication (Okuda et al., 2000). They have been also described to play roles during trafficking of proteins across the nuclear membrane (Borer et al., 1989), and regulation of apoptosis (Ahn et al., 2005; Ye, 2005). In addition, they have been shown to interact with transcription factors (Chen et al., 1994; Lin et al., 2006), and histone modifiers (Zou et al., 2008) to modulate transcription process. For example, mammalian nucleophosmin/B23 inhibits the DNA-binding and transcription activation of interferon regulatory factor-1 (IRF-1), which is a tumor suppressor (Kondo et al., 1997). One interesting note is that CK2 has been reported to phosphorylate nucleoplasmin to regulate the trafficking of nucleoplasmin from cytoplasm to nucleus (Vancurova et al., 1995).

To date, functional studies of *Drosophila* nucleoplasmin has been limited to in vitro analysis, which suggests that *Drosophila* nucleoplasmin displays similar activities, such as chromatin decondensation and assembly of nucleosomes, that are exhibited by its homologs from other species (Crevel et al., 1997; Ito et al., 1996b). It is detected at steady level throughout *Drosophila* development with the highest amount during early

Table 4-1: Identification of M8SD-CtD interacting proteins

Name	CG	Functions	Processes
Nucleoplasmin	CG7917	Nuclear chaperon Histone binding Nucleic acid binding	Nucleosome positioning/assembly Nuclear protein trafficking
Muscle LIM protein	CG1019	Zinc-ion binding Protein binding Scaffold for multi-complex	Cell differentiation Skeletal muscle development
Suppressor of white-apricot	CG3019	RNA binding mRNA splicing factor of <i>white-apricot</i>	Nuclear mRNA splicing RNA processing Regulation of gene expression
Ribosomal protein L6	CG11522-	Nucleic acid binding Structural constituent of large ribosome subunit	Spindle elongation Translation Mitotic spindle organization & biogenesis
Ribosomal protein L21	CG12775	Structural constituent of large ribosome subunit Nucleic acid binding	Translation Mitotic spindle elongation Mitotic spindle organization
Ribosomal protein S4	CG11276	RNA binding Structural constituent of small ribosome	Translation Mitotic spindle elongation Mitotic spindle organization
Fat body protein 1	CG17285	Protein transporter Oxygen transporter	Storage protein import into fat body
CG9568	CG9568	N/A	N/A
Salivary gland secretion 3	CG11720	Structural molecule	Puparial adhesion
Larval serum protein 1 beta	CG4178-	Nutrient reservoir Oxygen transporter	Transport
CG3589	CG3589	Serine-type endopeptidase	Proteolysis
Beta-4-galactosyltransferase 7	CG11780	Xylosylprotein 4-beta-galactosyltransferase activity Galactosyltransferase activity; metal ion binding	Proteoglycan biosynthetic process Glycosaminoglycan biosynthetic process Chondroitin sulfate biosynthetic process Heparan sulfate proteoglycan biosynthetic process

BLAST analysis of 14 cDNA's isolated from a pool of 1080 interacting clones. Functions and involved processes of each protein were collected from online databases such as Flybase, BioGrid, Entrez-Gene, InterPro, and Panther, and peer-reviewed research papers. Each sequenced plasmid was isolated from independent clones, but some of them encode the same proteins (such as salivary gland secretion 3 and CG3589). For duplication, only of them is listed. No information was available for CG9568.

embryogenesis (Kawasaki et al., 1994). Phylogenetic studies indicate *Drosophila* CG7911 to have high sequence homology to *Drosophila* nucleoplasmin and other NPM proteins, but it has not been characterized (Eirín-López et al., 2006).

Muscle LIM protein at 84B

Muscle LIM protein at 84B (Mlp84B) contains a functional domain called LIM, which consists of a zinc-binding motif. The LIM motif functions as a multiple-binding and adaptor scaffold that recruits interacting proteins into multi-component complexes to facilitate their interactions (Dawid et al., 1998; Kadrmas and Beckerle, 2004; Schmeichel and Beckerle, 1994). Specifically, Mlp84B belongs to the cysteine-rich protein (CRP) sub-family within the LIM family, which has been shown to function during cell differentiation and proliferation during development (Dawid et al., 1998) (for reviews, see (Weiskirchen and Günther, 2003). CRP proteins also promote and stabilize protein assembly on actin-based cytoskeleton.

In vertebrates, there are three conserved isoforms of CRP (CRP1, CRP2, CRP3/MLP), which have been implicated in cardiac (Jain et al., 1998) and skeletal muscle cell differentiations (Arber et al., 1994; Weiskirchen et al., 1995), and thymus LIM protein (TLP) (Kirchner et al., 2001). Muscle LIM protein (MLP) has been shown to interact with bHLH transcription factors such as MyoD, myogenin and MRF4 to regulate transcription of muscle-specific target genes during myogenic differentiation (Kong et al., 1997).

Drosophila has two isoforms, Mlp84B and Mlp60A, which are highly homologous (~50%) and share many of the features of their mammalian homologs (Stronach et al., 1996). Expression of Mlps has been reported during muscle development, and its level peak late in embryogenesis and during metamorphosis, two steps during which specifications and differentiation occur (Stronach et al., 1996). Studies indicate that Mlp functions during mesoderm specification and patterning, and is concomitant with terminal

muscle differentiation. Also, Mlp is reported to be a component of muscle cytoarchitecture (Stronach et al., 1999).

CRP-like proteins have been also identified in protozoa (Prassler et al., 1998) and plants (Eliasson et al., 2000; Mundel et al., 2000), which underscores evolutionarily conserved functions of CRP proteins throughout species.

Suppressor of white-apricot (SWAP)

Suppressor of white-apricot (SWAP) is a regulator of alternative splicing that is known to be involved in the splicing of *white-apricot* mRNA and its own mRNA (Zachar et al., 1987; Zachar et al., 1993). Mutants of *suppressor of white-apricot* display eye color mutations in *Drosophila*, which implicate the role of SWAP in eye color formation during development (Zachar et al., 1987). Three other regulators of splicing are known in *Drosophila*, which include Sex-lethal, Transformer, and Transformer-2 that control the alternative splicing of transcription factor Doublesex during sex determination (Lopez, 1998). Alternative splicing as post-translational regulation is increasingly reported in many different contexts. It has been implicated in regulation of tissue-specific gene expressions during cell differentiation, by regulating DNA-binding affinity and specificity of transcription factors (López, 1995). In addition, it is also reported to play a role during apoptosis (Jiang and Wu, 1999).

4.6. Future studies

The two-hybrid assay has identified a number of proteins that interact with M8SD-CtD. However, the potential implications/roles of their interactions with M8 remain to be investigated, because the two-hybrid screen only detects the interaction, not its role. Future studies will be required to delineate whether these interactions are physiologically relevant, and the consequences of these interactions on repression by E(spl)M8 in vivo. Such studies

could involve mapping of the interaction domains, the requirement for phosphorylation by CK2, and the most importantly whether mutations in the genes encoding these interacting proteins modulate repression by ectopically expressed M8. It is important to underscore, that neither nucleoplasmin nor LIM proteins have been previously shown to function during neurogenesis. However, it is becoming increasingly apparent that inhibitory Notch signaling and repression by E(spl) members also play roles in other developmental processes. Of note is the role of Notch and E(spl) during myogenesis, suggesting that the E(spl) repressors may be deployed during any binary cell-fate determination.

5. Conclusions

A two-hybrid screen of *Drosophila* embryonic cDNA library was conducted using M8SD-CtD as bait. In the assay, the interaction between the bait plasmid and the target cDNA led to induction of *HIS3* and *LacZ* reporter genes. From a total of 3.0×10^7 transformants, 1080 interacting clones who exhibited HIS^+ and $LacZ^+$ were isolated. The screen has identified a number of interacting partners including nucleoplasmin, suppressor of white-apricot, and muscle LIM protein. The functional roles, if any, of their interaction with M8 will require further investigations.

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Appendix 1: List of Primers

Primer	Sequence	Description	Purpose
R1KozIFM5	5'- gg <u>GAA TTC AAC</u> <i>atg</i> GCA CCA CAG AGC AAC AAC AGC -3'	Forward primer <u>GGATTC</u> =EcoRI; CAAC =Kozac; <i>atg</i> =Start codon; 21 base pairs complementary to 5' end of <i>m5</i> cDNA	To PCR amplify <i>m5</i> cDNA from <i>LexA-m5</i> while adding EcoR1 and Kozac sites at 5' UTR.
M5Xho1P1	5'- cc <u>CTC GAG</u> <i>tta</i> CCA AGG GCG CCA CAT GG - 3'	Reverse primer <u>CTCGAG</u> =Xho1 site; <i>tta</i> =stop codon; 20 base pairs complementary to 3' end of <i>m5</i> cDNA	To PCR amplify <i>m5</i> cDNA from <i>LexA-m5</i> while adding Xho1 site at 3' UTR
m5SA-P1	5'- CC TCG GGA TAC CAC <u>GCC</u> GAT AAC GAG GAC TC -3'	Forward primer GCC = Ala (chosen based on codon bias)	SDM (Ser156Ala) on pBS(KS+)-m5 to generate pBS(KS+)-m5SA
M5SA-P2	5'- GA GTC CTC GTT ATC <u>GGC</u> GTG GTA TCC CGA GG -3'	Reverse primer GGC =Ala (chosen based on codon bias)	SDM (Ser156Ala) on pBS(KS+)-m5 to generate pBS(KS+)-m5SA
m5SD-P1	5'- CC TCG GGA TAC CAC <u>GAC</u> GAT AAC GAG GAC TCT C -3'	Forward primer GAC =Ser (chosen based on codon bias)	SDM (Ser156Asp) on pBS(KS+)-m5 to generate pBS(KS+)-m5SD
M5SD-P2	5'- G AGA GTC CTC GTT ATC <u>GTC</u> GTG GTA TCC CGA GG -3'	Reverse primer GTC =Ser (chosen based on codon bias)	SDM (Ser156Asp) on pBS(KS+)-m5 to generate pBS(KS+)-m5SD
m5*-P1	5'- GTC GAG TTC CAG CGC ATG <u>TAG</u> CAG GCC GAT CAG GTC CAG -3'	Forward primer TAG =Stop codon	SDM (Leu130Stop)on pBS(KS+)-m5 to generate pBS(KS+)-m5*
m5*-P2	5'- CTG GAC CTG ATC GGC CTG <u>CTA</u> CAT GCG CTG GAA CTC GAC -3'	Reverse primer CTA =Stop codon	SDM (Leu130Stop) on pBS(KS+)-m5 to generate pBS(KS+)-m5*
THS-AD1	5'-ATA CCA CTA CAA TGG ATG ATG-3'	Forward primer binds Gal4DB (5' of pACT- MCS)	PCR to eliminate Gro- coding cDNA in the library screen Sequencing the isolated cDNA's
THS-Gro	5'- CGC GGT GAG TGG GAT TCC ATT TCA TTT GCA -3'	Reverse primer binds AA-717 to AA-746 of Gro ORF	PCR to eliminate Gro- coding cDNA in the library screen

Appendix 2: List of plasmids

Plasmids	Construct; Host; Clone#	Note
pBSII(KS+) m5	EcoR1-KozIF-[m5-ORF]-Xho1 in pBSII(KS+) DH5 α Cln#7B	m5 cDNA was PCR amplified from LexA-m5 using primers R1KozIFM5 and M5Xho1P1 sequenced
pBSIIKS(+)-m5SA	EcoR1-KozIF-[m5-S156A-ORF]-Xho1 DH5 α Cln#1A	SDM from pBSII(KS+)m5 using primers m5SA-P1 and M5SA-P2 sequenced
pBSIIKS(+)-m5SD	EcoR1-KozIF-[m5-S156D-ORF]-Xho1 DH5 α , Cln#4A	SDM from pBSII(KS+)m5 using primers m5SD-P1 and M5SD-P2 sequenced
pBSII(KS+)-m5*	R1-KozIF-[m5*-ORF (stop codon at Leu130)]-Xho1 in pBSII(KS+) DH5 α , Cln#4A	SDM from pBSII(KS+)m5 using primers m5*-P1 and m5*-P2 sequenced
pJG4-5(m5)	R1-KozIF-[m5-ORF]-Xho1, DH5 α Cln#A1.1 & A6.1	construct excised from pBSII(KS+)m5 with R1 and Xho1
pJG4-5(m5SA)	R1-KozIF-[m5-S156A-ORF]-Xho1 DH5 α Cln#B1.1 & B2.1	construct excised from pBSII(KS+)m5SA with R1 and Xho1
pJG4-5(m5SD)	R1-KozIF-[m5-S156D-ORF]-Xho1 DH5 α Cln#C9.1 & C12.1	construct excised from pBSII(KS+)m5SD with R1 and Xho1
pJG4-5(m5*)	R1-KozIF-[m5*-ORF (stop codon at Leu130)]-Xho1 DH5 α Cln#C2.1 & C3.1	construct excised from pBSII(KS+)-m5* with R1 and Xho1
pZEX (m5SA)	R1-KozIF-[m5-S156A-ORF]-Xho1 DH5 α Cln#A1.1 A2.1	construct excised from pBSII(KS+)m5SA with R1 and Xho1
pZEX (m5SD)	R1-KozIF-[m5-S156D-ORF]-Xho1 DH5 α Cln#B1.1 & B2.1	construct excised from pBSII(KS+)m5SD with R1 and Xho1
pZEX(m5*)	R1-KozIF-[m5*-ORF (stop codon at Leu130)]-Xho1 DH5 α Cln#D2.1 & D5.1	construct excised from pBSII(KS+)-m5* with R1 and Xho1
pUAST(m5)	R1-KozIF-[m5-ORF]-Xho1 DH5 α Cln#C4.1 & A4.2	construct excised from pBSII(KS+)m5 with R1 and Xho1

Plasmids	Construct; Host; Clone#	Note
pUAST(m5SA)	R1-KozIF-[m5-S156A-ORF]-Xho1 DH5 α Cln#D1.1 & D3.1	construct excised from pBSII(KS+)-m5SA with R1 and Xho1
pUAST(m5SD)	R1-KozIF-[m5-S156D-ORF]-Xho1 DH5 α Cln#E2.1 & E3.1	construct excised from pBSII(KS+)-m5SD with R1 and Xho1
pUAST(m5*)	R1-KozIF [m5*-ORF (stop codon at Leu130)]-Xho1 DH5 α Cln#B2.1 & B3.1	construct excised from pBSII(KS+)-m5* with R1 and Xho1
pZEX(m5)	R1-KozIF-[m5-ORF]-Xho1 BL21+pTRX Cln# a & b	plasmid isolated from pZEX(m5) and transformed into BL21+pTRX <i>E. coli</i> strain for protein extraction
pZEX(m5SA)	R1-KozIF-[m5SA-ORF]-Xho1 BL21+pTRX Cln#A1.1a & A1.1b	plasmid isolated from pZEX(m5SA) and transformed into BL21+pTRX <i>E. coli</i> strain for protein extraction
pZEX(m5SD)	R1-KozIF-[m5SD-ORF]-Xho1, BL21+pTRX Cln#B2.1a & B2.1b	plasmid isolated from pZEX(m5SD) and transformed into BL21+pTRX <i>E. coli</i> strain for protein extraction
pGBT9-m8CT	R1-KozIF-[m8CT]-BamH1 DH5 α	construct excised from pBSII(SK+)-m8CT with R1 and BamH1
pGBT9-m8SDCT	R1-KozIF-[m8SDCT]-BamH1 DH5 α	construct excised from pBSII(SK+)-m8SDCT with R1 and BamH1
LexA-M8-CT	R1-KozIF-[m8-CT]-BamH1 DH5 α	construct excised from pBSII(SK+)-m8CT with R1 and BamH1
LexA-M8SD-CT	R1-KozIF-[m8SD-CT]-BamH1 DH5 α	construct excised from pBSII(SK+)-m8SDCT with R1 and BamH1