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Role of multi-site phosphorylation in regulation of E(spl) M8 activity during *Drosophila* neurogenesis

Mohna Bandyopadhyay

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Role of multi-site phosphorylation in regulation of E(spl)

M8 activity during *Drosophila* neurogenesis

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Dissertation submitted to the
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at West Virginia University
in partial fulfillment of the requirements for the degree of

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In
Biological Sciences

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ABSTRACT

Role of multi-site phosphorylation in regulation of E(spl) M8 activity during *Drosophila* neurogenesis Mohna Bandyopadhyay

Notch signaling is an evolutionarily conserved pathway that regulates cell-fate determination at various stages of development in invertebrate and vertebrate species. The Notch pathway mediates juxtacrine signaling through a process termed as lateral inhibition by which two equipotent cells adopt distinct cell fates. The functions of Notch have been exceptionally well analyzed during neurogenesis in *Drosophila*, particularly during eye and mechanosensory bristle morphogenesis. At the onset of neurogenesis, proneural bHLH activators encoded by *atonal* (*ato*) or the *achaete-scute complex* (*ASC*) is expressed in groups of cells, called proneural clusters (PNCs) in the eye and the bristle tissue respectively. From each PNC, however, only a single cell becomes the neural progenitor, the sensory organ precursor (SOP). This selection is mediated through E(spl) repressors, a group of evolutionary conserved bHLH repressors and the terminal effectors of the Notch pathway. Accumulating evidence suggest that repression of Ato/ASC by E(spl) occurs in a phosphorylation dependent manner. Protein kinase CK2 phosphorylates E(spl)-M8, which then undergoes a conformational change and antagonizes the proneural proteins. The work described in this dissertation aims to provide a more detailed understanding of the mechanism of neural repression by M8. The work described in Chapter 2 provides *in vivo* evidence that multi-site phosphorylation of the C-terminal domain (CtD) of M8 may be involved in regulation of repressor activity. Using phospho-mimetic variants of M8 we have shown that both CK2 and MAPK motif in the CtD may be modulated for the protein to exhibit neural repression. Multisite phosphorylation may act as a 'switch' controlling the activity and onset of repression by M8 during neurogenesis. The studies in Chapter 3 demonstrate a direct role of P-domain, a conserved phosphorylation domain of CtD, in autoinhibition model of M8. The deletion variant studies provide strong evidence that the P-domain provides critical autoinhibitory contact with HLH and/or Orange domain to regulate repression of Ato/ASC. This study provides a fundamental reinterpretation of the mechanism by which truncated protein, M8* encoded by *m8* allele *E(spl)D*, that lacks the autoinhibitory domain, elicits precocious Ato antagonism thus interfering with first phase of Notch signaling and perturbing eye development. Our studies also implicate P-domain as a target of Slmb, a subunit of E3 ubiquitin ligase that may give rise to 'phosphodegrom' necessary for proteasomal degradation and rapid clearance of M8. Together, the studies described in this dissertation provide a mechanism of M8 regulation by posttranslational regulation to elicit the effects of inhibitory Notch signaling during lateral inhibition.

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Table of Contents

ABSTRACT-----	ii
Acknowledgments-----	iii
List of Figures -----	vi
CHAPTER 1: Introduction-----	1
The Notch Pathway-----	3
Notch Pathway and diseases -----	5
Components of the Notch pathway-----	6
Structure of Drosophila Notch -----	7
Notch Ligands-----	8
Regulation of Notch Signaling -----	9
Receptor maturation-----	9
Ligand Binding and Receptor Activation -----	10
Target gene expression -----	11
Eye and bristle development in Drosophila-----	12
Notch Signaling and Lateral Inhibition -----	12
Notch Signaling during Drosophila eye development-----	13
Notch Signaling during Drosophila bristle development-----	15
E(spl)-C complex and bHLH repressors -----	17
Role of E(spl) members in eye and bristle development-----	19
Regulation by E(spl) repressors is phosphorylation dependent-----	20
The ‘autoinhibition’ Paradox-----	22
Protein Kinase CK2-----	23
Biochemistry of CK2-----	24
CK2 and its role in development -----	25
Drosophila CK2-----	26
The Epidermal Growth Factor Receptor (EGFR) Pathway -----	28
EGFR Receptor and Ligands -----	28
EGFR pathway activation -----	30
Role of EGFR in Drosophila eye and bristle development -----	32
Suspected role of EGFR signaling during R8 specification in Drosophila -----	33
Preface to chapters 2-4 of this dissertation-----	34
CHAPTER 2: The conserved MAPK site in the Notch effector E(spl) M8 controls repressor activity in the eye-----	45

Abstract -----	46
Introduction -----	47
Results -----	51
M8 repressor activity in the developing eye requires multi-site phosphorylation -----	51
E(spl)-M8 harbors a highly conserved consensus site for MAPK-----	53
An intact MAPK site is essential for activity of the CK2 mimic M8-S159D -----	54
M8 variants with a modified MAPK site perturb R8 specification -----	55
An Asp residue at the MAPK site elicits inappropriate earlier activity of M8 -----	56
Altered EGFR signaling modulates M8 variants with an intact MAPK site -----	57
Deletion of the CK2 or MAPK site mimics Asp-variants of M8 at these two kinase sites -----	58
PP2A may target the MAPK site during M8 regulation -----	59
Discussion -----	60
Multi-site phosphorylation exerts spatial control over M8 activation in the eye -----	60
Notch and the specification of the R8 cells -----	61
EGFR and the specification of the R8 cells -----	62
Materials and Methods-----	65
CHAPTER 3: Identify the role of P-domain in autoinhibition of M8-----	82
Abstract -----	83
Introduction -----	84
Results -----	88
Deletion variants and their differential roles at stage-1 of the MF -----	88
All three-deletion variants elicit eye defect at stage-2/3 of the MF -----	89
M8 deletion variants perturb R8 specification and bristle formation-----	90
The effects of M8- Δ CK2 are sensitive to altered EGFR levels -----	91
M8-CtD strongly suppresses the eye and bristle defects of all the deletion variants -----	92
P-domain in M8 may be a target for modulation by Slmb-----	93
Discussion -----	94
Materials and Methods-----	99
BIBLIOGRAPHY-----	113

List of Figures

Fig. 1: Core components of Notch pathway and Drosophila Notch Receptor.	36
Fig. 2: Schematic representation of Notch signaling pathway.	37
Fig. 3: Lateral inhibition during R8 specification in Drosophila eye development.	38
Fig. 4: Compound eye of Drosophila and mechanism of R8 specification and patterning.	39
Fig. 5: Bristle morphogenesis in Drosophila.	40
Fig. 6: E(spl) C locus and its molecular synteny across 12 Drosophila species.	41
Fig. 7: Structure of M8 and autoinhibition model of M8 regulation.	42
Fig. 8: Structure of CK2 holoenzyme and the alleles of Drosophila CK2 α subunit.	43
Fig. 9: Components of the Drosophila EGFR pathway.	44
Fig. 10: MF specificity of M8-S159D.	68
Fig. 11: Conservation of Ser residues in the P-domain of E(spl)-M8.	69
Fig. 12: MAPK refractory but CK2 mimic does not perturb the eye.	71
Fig. 13: Secondary phosphorylation enhances M8 activity.	72
Fig. 14: Phosphomimics perturb R8 birth and differentiation.	73
Fig. 15: MAPK mutation causes precocious M8 repressor activity at stage-1 of MF.	74
Fig. 16: Decreased EGFR levels rescue reduced eye of CK2 mimic only.	76
Fig. 17: Deletion variants of M8 perturb eye development.	78
Fig. 18: MAPK site in M8 may be a target for modulation by PP2A.	80
Fig. 19: Phosphorylation and dephosphorylation regulates M8 repressor activity during retinal neurogenesis.	81
Fig. 20: Deletion variants and their MF specificity.	102
Fig. 21: Deletion variants enhance M8 activity at stage-2/3 of MF.	103
Fig. 22: Perturbation of R8 birth and differentiation and bristle formation at stage-2/3 of MF.	104
Fig. 23: Decreased level of EGFR rescue reduced eye of M8- Δ CK2 only.	105
Fig. 24: M8-CtD suppresses the eye and bristle defects of deletion variants.	107
Fig. 25: P-domain in M8 may be a target for modulation by Slmb.	108
Fig. 26: Deletion variants perturb the eye at stage-4 of the MF.	110
Fig. 27: Epistatic model of M8 activity in the MF.	112

CHAPTER 1: Introduction

The development of single fertilized egg into a properly formed multicellular (metazoan) organism is one of biology's deepest and yet to be fully understood enigmas. This, in large part, reflects the necessity for multiple (combinatorial) approaches such as genetics, cell biology, biochemistry and biophysics, all of which are essential to understand how a single totipotent cell (the fertilized egg) undergoes precise and progressive changes in cell fates to give rise to a properly formed individual animal. Remarkably, the developmental processes, which dictate the diverse cell fates, hinge upon only a handful of signaling pathways that are highly conserved through evolution. These signaling pathways ensure that cell proliferation, differentiation, migration, apoptosis, etc., are coordinated in a highly precise manner during tissue formation, patterning and organogenesis.

The five conserved signaling pathways that are key to development are Notch (N), Epidermal Growth Factor Receptor (EGFR), Decapentaplegic (Dpp), Hedgehog (Hh) and Wingless (Wg). Over the years, analyses in various experimental model organisms have outlined the core components and the architecture of these signal transduction pathways. Nevertheless, the challenge before us is to understand how these pathways control the formation of various tissues and organs, in light of the fact that they play reiterative roles throughout development.

In the past few decades, remarkable progress has been made in our understanding of the mechanisms underlying cell-cell signaling. It is, perhaps, not surprising that perturbations of these signaling pathways, either through inappropriate activation or inactivation, underlie various birth defects and human disease states. In addition, it is becoming increasingly appreciated that polymorphisms in the components of these signaling pathways manifest in numerous diseases with 'sporadic' (tissue-specific) incidence. It is therefore important to understand the precise spatial as well as temporal coordination between these biochemical and genetic circuits that regulates development, and this aspect

remains a major challenge before us. This understanding is important to decipher tissue-specific molecular mechanisms that underlie developmental disorders and disease states.

Germane to the studies described in this dissertation (Chapters 2-4, see below), this introduction focuses on two of these signaling pathways, i.e., Notch and EGFR, and concludes with a discussion of the impact of phosphorylation on one (of several) effectors of Notch signaling, a mechanism enabling the intersection of these two pathways via post-translational regulation. This introduction reviews the conserved components of these pathways, their signaling logic, their roles in two well-studied developmental paradigms, and concludes with a section outlining gaps in our current knowledge. Because these pathways coordinate cell fate specification throughout development, this review focuses on development of the nervous system in arguably the preeminent genetic model, the fruit fly *Drosophila melanogaster*.

Studies in *Drosophila* have, over more than a hundred years, served as a paradigm and a springboard for understanding not only general principles of animal development, but have provided some of the most sophisticated insights into how cell signaling controls cell fate specification. In part this reflects a short generation time, the low expense of maintaining fly stocks, a large collection of mutants, the ease of transgenic analysis, and a battery of molecular, genetic and immunological resources, all housed at repositories that are widely available to the research community.

The Notch Pathway

The existence of the Notch locus was revealed by the work of John S. Dexter in 1914, upon the identification of the (name giving) mutation called 'Notch'. He described the mutation as a heritable abnormality in fruit flies, characterized by 'notching' of the tips of their wings. Later, Thomas Hunt Morgan, through studies of mutations in *Drosophila*, identified the first allele of the *Notch* gene. The nomenclature was based on the 'notched'

wing phenotype associated with the loss of wing tissue. Although the gene was identified, its role in development remained largely obscure until 25 years later when David Poulson reported that the complete loss of *Notch* function resulted in embryonic lethality (Poulson, 1937). His work on *Notch* mutants was the first to associate a gene whose activity was specific for development in an organism.

Subsequent studies led to the discovery of additional genetic loci that were necessary for proper Notch function, and the realization that aberrant Notch signaling elicits neurogenic phenotypes (see below). The first detailed insights into the mode of action of Notch signaling came from studies in *Drosophila melanogaster* and *Caenorhabditis elegans*, which paved the way for better understanding the underlying mechanisms in higher eukaryotes, including mammals (Greenwald, 1998).

As is the case for many genes, the number of Notch receptor subtypes differs widely between species. While *Drosophila* has a single Notch receptor, *C. elegans* has two genes, which code for Notch receptors, LIN-12 and GLP-1 (Greenwald et al., 1983). In contrast, mammals have four distinct Notch receptors, Notch 1-4 (del Amo et al., 1993; Lardelli et al., 1994; Uyttendaele et al., 1996; Weinmaster et al., 1992). It should, however, be noted, that although the number of receptors may vary amongst the aforementioned taxonomic groups, the basic mechanism of Notch signaling appears to be conserved (see below). As a focus of the studies described in this introduction, the mechanism of Notch function will be discussed mainly in the developmental context of *Drosophila* as a model system.

The Notch signaling pathway is involved throughout development, and regulates neurogenesis, myogenesis, somitogenesis, oogenesis, angiogenesis, left-right symmetry, formation of the cardiac and renal systems, and stem cell maintenance (Artavanis-Tsakonas et al., 1999; Lai, 2004; Mumm and Kopan, 2000). More recent studies are revealing that Notch plays additional roles in the adult, such as its impact on the circadian rhythms.

Despite these diverse contexts, this pathway mediates short-range (juxtacrine) signaling between adjacent cells, and in an exclusively contact-dependent manner because both the receptor and its ligands are membrane-bound proteins. This is underscored by the findings that soluble forms of the ligand Delta have the capacity to interact with the receptor, but are unable to mediate Notch signaling, and trigger tumorigenicity in cultured mammalian fibroblasts (Li et al., 2007; Urs et al., 2008).

The Notch receptor signals through three primary modes. The first of these, lateral inhibition, is the process by which Notch inhibits the default cell fate from a group of equipotent cells. Consequently, lateral inhibition is necessary to ensure that from each cluster of equipotential cells only a single cell is allowed to maintain the primary cell fate. This process, the focus of my dissertation research, is discussed in greater details later in this introduction. The second mode of Notch signaling is in cell fate specification, in part by its role in asymmetric cell division. During asymmetric cell division, only one cell inherits a Notch regulator. As a result, the two resulting daughter cells display differential Notch activities, which, in turn, confer distinct cell lineages/fates. The third, boundary formation, occurs when a population of cells induces Notch activity in nearby cells that leads to their segregation into two distinct cell types. Although classically associated with binary cell fate decisions, and in addition to the three modes of signaling mentioned above, Notch is also involved in regulation of cell proliferation, maintenance of stem cells as well as apoptosis (Artavanis-Tsakonas et al., 1999).

Notch Pathway and diseases

Given that Notch functions in diverse developmental contexts, it is perhaps unexpected that mutations affecting Notch signaling elicit tissue/organ-specific congenital abnormalities and have been linked to specific disease states. These include diseases affecting various organs such as the liver, eye, kidney, vasculature and heart. Some of the

inherited syndromes associated with abnormal Notch signaling include Alagille syndrome (Artavanis-Tsakonas et al., 1999; Gridley, 2003; McCright et al., 2002), characterized by organ and skeletal abnormalities, CADASIL, a disorder characterized by stroke and dementia (Joutel et al., 1996; Penton et al., 2012), spondylocostal dysostosis, a skeletal abnormality (Sparrow et al., 2008), T-ALL, a T-cell acute lymphoblastic leukemia (Weng et al., 2004), Hajdu-Cheney syndrome, characterized by severe and progressive bone loss (Simpson et al., 2011), and neurodegenerative diseases such as Batten syndrome (Colombo et al., 2013; John et al., 2002). In addition, defects in this pathway have been linked to congenital heart diseases, the hematopoietic malignancy multiple myeloma, multiple sclerosis, familial aortic valve disease, and other cancers (Garg et al., 2005; Garside et al., 2013; High and Epstein, 2008; John et al., 2002). The broad roles of Notch signaling and the wide spectrum of disorders manifesting upon aberrant signaling makes it likely that the 'threshold' of Notch signaling required for its diverse tissue-specific functions is distinct both quantitatively and/or qualitatively, which has previously been implicated in studies on Notch in the *Drosophila* model organism (see below). Surprisingly, some of the aforementioned developmental abnormalities are not fully recapitulated in the mouse model, suggesting that our understanding of this pathway is still incomplete. It would seem that studies in both invertebrate and vertebrate model systems are needed to illuminate complexities in Notch signaling to impact basic and/or clinical research relevant to Notch-related pathologies.

Components of the Notch pathway

The core components of the Notch pathway were identified through genetic analyses in *C. elegans* and *Drosophila*. The pathway has a simple architecture where all the major components are conserved from *Drosophila* to mammals (Fig. 1A). In *Drosophila*, the pathway consists of a transmembrane Notch receptor, its ligands Delta (Dl) or Serrate (Ser),

the downstream transcription factor Suppressor of Hairless (Su(H)), and the final effectors- the basic Helix-Loop-Helix (bHLH) transcription repressors encoded by the *Enhancer of split Complex (E(spl)C)* (Delidakis and Artavanis-Tsakonas, 1991; Schweisguth and Posakony, 1992). The *E(spl)C*, which is located on the third chromosome, encodes a family of structurally similar bHLH repressors called Mδ, Mβ, Mγ, M3, M5, M7 and M8 and their common co-repressor Groucho (gro) (Delidakis and Artavanis-Tsakonas, 1991; Hartley et al., 1988; Klambt et al., 1989). The E(spl) proteins function as repressors; this requires the formation of homo/hetero-dimers as well as interaction with Gro (Alifragis et al., 1997; Giebel and Campos-Ortega, 1997)kuzbanin. While the mechanistic reason for the Gro-dependency is well understood, the reason(s) why dimerization is critical to repression remain unclear.

Structure of *Drosophila* Notch

The Notch receptor in *Drosophila* is a 300 KDa single-pass transmembrane receptor (Fig. 1B). The extracellular domain contains a series of Epidermal Growth Factor (EGF)-like repeats that are responsible for ligand binding. The Notch receptors of different animals vary in the number of EGF-like repeats. Each EGF repeat is approximately 40 residues long and contains six cysteine residues that are held together by three disulfide bonds (Fleming, 1998). The fruit fly Notch receptor has 29-36 EGF-like repeats (Fleming, 1998). The EGF-like repeats undergo a series of modifications via glycosylation, which are critical for receptor-ligand interactions and are thought to influence signaling strength. The minimal region of the *Drosophila* Notch receptor, which is essential and sufficient for binding to the ligands Delta and Serrate encompass the EGF-like-repeats 11 and 12 (Rebay et al., 1991; Xu et al., 2005)

Three highly conserved cysteine rich LIN-12/Notch repeats, the LNRs, follow the EGF-repeats. The LNR is followed by a heterodimerization (HD) domain, which is adjacent

to the transmembrane domain (Tien et al., 2009). The HD domain harbors the S2 cleavage site, which helps to maintain the receptor in a metalloprotease-resistant conformation (Gordon et al., 2007). Together, the LNRs and the HD domains sandwiched between the ligand binding and the transmembrane regions of the protein comprise the negative regulatory region (NRR), which helps to maintain Notch in a resting confirmation (Fig. 1B).

The cytoplasmic region of Notch contains several functional domains, represented by an N-terminal recombination binding protein-J associated molecule (RAM) domain (Tamura et al., 1995) followed by an ankyrin (ANK) domain and culminates in a long unstructured linker that includes a variable trans-activation domain (TAD) (Kurooka et al., 1998). There are seven ANK repeats, which represent the most conserved region of the cytoplasmic region of the receptor. Each ANK repeat is comprised of 33 residues and together make up an essential domain for Notch-receptor function. At the extreme end of C-terminal region is a conserved proline/glutamic acid/serine/threonine-rich (PEST) motif that functions as a degradation signal and regulates receptor stability (Kopan and Ilagan, 2009; Moretti and Brou, 2013) (Fig. 1B).

Notch Ligands

Since Notch signaling is dependent on direct cell-to-cell interaction, the canonical ligands that bind and activate the Notch receptors are single-pass transmembrane proteins characterized by the presence of an N-terminal (NT) domain, followed by a DSL (Delta, Serrate, LAG-2) domain and a varying number of EGF-like repeats (Chillakuri et al., 2012). The canonical Notch ligands belong to two classes, the Delta/Delta-like and the Serrate/Jagged class. The Serrate/Jagged class of ligands has an additional domain of cysteine rich repeats close to the transmembrane domain. *Drosophila* has two canonical Notch ligands- Delta and Serrate. In contrast, there are five canonical Notch ligands in mammals- Jagged-1, Jagged-2, DLL1, DLL-3 and DLL-4 (D'Souza et al., 2010).

Regulation of Notch Signaling

Since the Notch pathway regulates numerous cell-fate specification events in metazoan animals, deregulation of signaling underlies multiple developmental disorders (see above). Hence, both the timing and strength of receptor activation are key steps to regulate the pathway. A host of post-translational modifications starting with ligand-mediated activation, receptor proteolysis and target selection are critical to the regulation of Notch signaling. A brief discussion on the molecular events of regulatory mechanism of Notch signaling is presented in the following section.

Receptor maturation

The Notch receptor undergoes three successive proteolytic cleavages in order to take the form of a mature, membrane-anchored receptor (Fig. 2). The first proteolytic cleavage, which is referred to as the 'S1 cleavage', occurs in the trans-Golgi complex and is facilitated by the Furin-like convertases (Logeat et al., 1998). The cleaved receptor is then targeted to the cell surface as a heterodimer composed of a Notch extracellular domain (NECD) and a Notch intracellular domain (NICD) held together by non-covalent interactions.

In addition to this first proteolytic cleavage, the receptor undergoes several glycosylation steps in the endoplasmic reticulum and the Golgi complex before it is targeted to the plasma membrane as a mature receptor (Fig. 2). The multiple EGF-like repeats of the extracellular domain are sites for glycosylation. After Notch is translated, the enzyme O-fucosyl transferase (O-Fut) adds the first fucose group to the EGF repeat 12 (Kopan and Ilagan, 2009). Initially it was thought that this particular modification is essential for the generation of a functional receptor (Okajima and Irvine, 2002). However, later studies in *Drosophila* demonstrated that non-fucosylated Notch was able to be trafficked properly to the cell membrane, interact with ligand and even able to mediate signaling (Okajima et al., 2008; Stanley, 2007; Vodovar and Schweisguth, 2008). Subsequently, additional

carbohydrate moieties are added to the fucosylated receptor, which is essential for Notch signaling. In the Golgi apparatus, Fringe catalyzes the transfer of N-acetylglucosamine (GlcNAc) to the EGF repeats of the Notch receptor (Haines and Irvine, 2003). Studies show that these glycosyl modifications alter the strength of ligand interaction with the Notch receptor and thus regulate its activity (as reviewed in vivo (Bray, 2006)). For example, Fringe-mediated glycosylation has been shown to increase the affinity of Notch receptor for the ligand Delta while reducing Serrate-binding (Bruckner et al., 2000; Xu et al., 2007). More recently, another glycosyltransferase, RUMI, has been identified that adds O-glucose to specific Ser residues in the extracellular domain of *Drosophila* Notch (Acar et al., 2008). Unlike other glycosyltransferases, loss of RUMI leads to impaired Notch signaling in a variety of developmental contexts, indicating that it is a general regulator of the Notch pathway.

Ligand Binding and Receptor Activation

Canonical Notch signaling occurs when a ligand of the DSL (Delta and Serrate in *Drosophila*, Jagged in mammals and LAG-2 in *C.elegans*) family present on one cell interacts with Notch receptors present in the adjacent cell. Ligand binding leads to activation of the receptor, which is regulated by a key proteolysis event at the S2 site within the LNR region. The ADAM family of metalloproteases mediates this S2 cleavage (Fig. 2). In the absence of the ligand, the LNR domain sterically hinders the HD domain to occlude the S2 cleavage site. Ligand binding to the receptor changes the conformation of the LNR region and exposes the S2 cleavage site, which is now available to ADAM proteases. ADAM 10 (also known as Kunzbanian in *Drosophila*) is responsible for cleaving Notch receptor at S2 site (Pan and Rubin, 1997). Additionally, endocytosis of Notch extracellular domain into the ligand-expressing cell provides a mechanical force that “pulls” away the protective LNR repeats from the HD domain to expose the S2 site (as reviewed in (Gordon

et al., 2008). This process ensures that Notch activation only takes place in two adjacent cells and only in the presence of ligand. This view corroborates the finding that soluble ligands are insufficient to activate Notch receptor in cell culture (Sun and Artavanis-Tsakonas, 1997). The S2 cleavage event is followed by further proteolysis at the S3 site in the Notch extracellular truncation domain (NEXT) mediated by γ -secretase, which releases the NICD (Okochi et al., 2002). Thus, regulated proteolytic events, ligand binding and endocytosis of Notch receptor domain are crucial steps in receptor activation.

Target gene expression

The NICD released after the γ -secretase proteolytic event induces target-gene transcription. Following its release, the NICD fragment translocates to the nucleus and interacts with the CSL (CBF-1 in mammals, Su(H) in *Drosophila* and LAG-1 in *C. elegans*) family of transcription factors via its RAM domain (Artavanis-Tsakonas et al., 1995; Bailey and Posakony, 1995). This interaction switches the CSL repressors into transcriptional activators, which then drive the transcription of the terminal effectors of the Notch pathway, the bHLH E(spl)/HES repressor proteins (Fig. 2). Prior to its activation by NICD, the CSL complexes with the co-repressors C-terminal binding protein (CtBP) and Groucho (Gro) and recruits Histone deacetylases (HDACs) to maintain E(spl)/HES in a repressed state (Barolo et al., 2002; Lai, 2002). NICD and CSL combine to recruit a co-activator such as Mastermind (Mam) in *Drosophila* or Mam-like (MAML) in mammals (Petcherski and Kimble, 2000). Mam in turn recruits histone acetyl transferases (HATs) and p300, which stabilize the transcription initiation complex and activates expression of target genes (Wallberg et al., 2002). Subsequent phosphorylation of the NICD-CSL-MAM transcriptional complex downregulates the activity and ensures degradation of the NICD. The PEST domain in NICD undergoes phosphorylation by CDK8 and is then targeted for proteasomal degradation by E3 ubiquitin ligase (as reviewed in (Kopan, 2012)). This leads to the

termination of the Notch signal, and in those cases where Notch acts repeatedly, resets the cell for the next round of signaling.

Eye and bristle development in *Drosophila*

The functions of Notch have been exceptionally well analyzed for neurogenesis (Artavanis-Tsakonas et al., 1999; Mumm and Kopan, 2000), during which binary cell fate is determined. The two external sensory organs of *Drosophila*, which have led to our understanding of Notch pathway in neurogenesis, include the compound eye and the mechanosensory bristles. The development of these sensory organs in a precise pattern initiates during the larval stage and is completed during the pupal stage of development. The cells of these two organs are generated from the organ-specific epithelial sheet (a monolayer) known as the imaginal disc, which undergoes sequential specification and differentiation achieved by spatially and temporally restricted gene expression of various transcription factors. These two sensory organs provide excellent readouts of Notch pathway activity as the bristles occupy invariant positions in the body and the repeating units of the compound eye (ommatidia or facets) are arranged in a precise hexagonal pseudo-crystalline array (Cagan and Ready, 1989; Cubas et al., 1991; Ready et al., 1976; Schweisguth et al., 1997; Simpson et al., 1999). Hence, any changes in the Notch pathway activity perturb the hexagonal arrangement of the ommatidia and thus the architecture of the eye, as well as the position and the number of bristles on the body. Both of these defects result in externally visible phenotypes and even subtle perturbations in these two sensory organs are easily discernible by light microscopy. This initial observation of perturbation of neurogenesis can be followed by immunohistochemistry on the eye-antennal disc or the wing disc, parts of which give rise to the adult eye and thorax, respectively.

Notch Signaling and Lateral Inhibition

At the onset of neurogenesis, proneural bHLH activators encoded by *atonal* (*ato*) or

the genes of the *achaete-scute complex* (ASC) are expressed in groups of cells, called the proneural clusters (PNCs) (Heitzler et al., 1996; Jarman et al., 1995; Jarman et al., 1994). The activities of these proneural proteins drive the formation of the PNCs, in which all cells have somewhat equal potential to adopt the neural fate (Dambly-Chaudiere and Vervoort, 1998). From each PNC, however, only a single cell will go on to become the neural progenitor, the sensory organ precursor (SOP) (Fig. 3). This restriction of the SOP-fate to a single cell from a PNC has been termed lateral inhibition (Simpson, 1990). During this process, one of the cells of PNC gains an advantage over the others by producing higher levels of Ato/ASC, and thereby expresses higher levels of DI. This asymmetry in DI levels elicits activation of Notch in the adjacent cells of the PNCs (the non-SOPs). Specifically, activation of Notch results in the cleavage and nuclear translocation of its intracellular domain (NICD), where it associates with and activates Su(H) leading to the E(spl) transcription (Bailey and Posakony, 1995) (Fig. 3). The E(spl) repressors along with Gro then antagonize Ato/ASC to restrict the SOP fate in all but one cell of a PNC. In fact, Gro is a corepressor essential for a large group of bHLH proteins; those encoded by the E(spl)C, Deadpan and Hairy (Paroush et al., 1994). Consistent with this model, decreased Notch pathway activity results in the emergence of multiple SOPs from each PNC. This supernumerary SOP specification results in a rough eye and ectopic and closely juxtaposed bristles.

Notch Signaling during *Drosophila* eye development

The remarkably precise hexagonal geometry of the compound eye of *Drosophila* has afforded an ideal system to understand the role of different signaling pathways in pattern formation. The Notch pathway is central to *Drosophila* eye development. The compound eye of *Drosophila* is composed of ~800 ommatidia arranged in a hexagonal lattice (Fig. 4). Each ommatidium is composed of eight photoreceptors (R1-R8- the retinula cells), twelve

accessory non-neuronal cells such as pigment cells and cone cells, and an interommatidial bristle (IOB). During the third larval stage (instar), eye morphogenesis initiates at the posterior margin of the eye-antennal disc, a monolayer neuro-epithelium that is the progenitor of the retina. Retinal neurogenesis starts with the specification of the R8 photoreceptors (Jarman et al., 1994). R8 specification occurs in a moving wave of differentiation, called the morphogenetic furrow (MF) (Fig. 4), which is discernible in unstained tissue due to constriction of the cells mediated by localized polarization of actin. R8 specification is a critical and essential first step during eye morphogenesis as it subsequently recruits all other cell types in the assembling ommatidia. In “null mutation” of *ato* (*ato*¹) no R8's are specified and the eye is lost. Because of this vital role played during ommatidial formation, R8 cells are referred to as the ‘founding’ photoreceptors. While R8 selection occurs within the MF, all secondary photoreceptor recruitment occurs posterior to it (Wolff and Ready, 1991). The movement of the MF from the posterior margin of the eye disc to its mid-point (its final limit) occurs in 48 hrs. Thus analyses of the mid-late L3 stage eye disc provide a broad temporal window to understand the mechanisms of specification of various cell types during neurogenesis.

During R8 ontogeny, Notch serves biphasic roles. At stage-1, the anterior margin of the MF, cells begin to express low levels of *ato* cell autonomously to form the ‘Ato-stripe’. (Fig. 4) This proneural enhancement step is induced by Notch activation (Baker et al., 1996). *Ato* then dimerizes with Daughterless (*Da*), eliciting high levels of *ato* transcription. This process called *ato* auto-activation generates pre-R8 clusters, the intermediate groups (IGs) with somewhat equal potential to become an R8. At stage-2/3 of the MF, a single R8 is non-autonomously selected from each IG by lateral inhibition (Fig. 4). During this process, Notch mediates expression of E(spl) proteins, which repress *Ato* in all but a single cell of an IG (Ligoxygakis et al., 1998). By stage-4, single patterned R8s emerge and these

cells are marked by the sustained expression of the Ato target gene *senseless* (*sens*) whose expression is first observed at the three-cell Ato positive cluster (Nolo et al., 2000; Pepple et al., 2008). After this point in R8 ontogeny, Sens expression continues to be maintained in all differentiated R8s throughout the developing eye disc. Posterior to the MF, R8s emerge in a precise phase-shifted manner, such that R8s of one column are out of phase to those in the adjacent column. This alternating spacing (patterning) is vital for the hexagonal architecture of the adult eye. Consistent with this view, targeted overexpression of Ato in the MF elicits the specification of supernumerary R8s, and results in a rough eye phenotype (White and Jarman, 2000). The R8s then systematically recruit surrounding uncommitted cells as the R2/R5, R3/R4, R1/R6 cells, concluding with the specification of the R7 cell (Cagan and Ready, 1989) (Fig. 4). All of these cell fate specifications also involve Notch signaling (Fanto and Mlodzik, 1999; Tomlinson and Struhl, 2001).

Notch Signaling during *Drosophila* bristle development

The external sensory organs of *Drosophila* also include mechanosensory bristles of the peripheral nervous system. The two types of bristles that have been studied extensively to understand neurogenesis are the macrochaetes and the interommatidial bristles (Fig. 5). The number and position of these bristles are invariant and are regulated by Notch-mediated lateral inhibition (Hartenstein and Posakony, 1990). Each bristle contains four distinct types of cells; a socket cell, a sheath cell, a shaft cell and a neuron.

Each bristle arises from a single precursor cell termed the sensory organ precursor (SOP), which is selected from a group of equipotent cells, the PNCs. Although SOP specification occurs at third instar larval stage, bristle morphogenesis does not begin until the pupal stage. Unlike the eye, however, expression of ASC proneural activators is independent of Notch signaling and instead depends on pre-pattern factors (Calleja et al., 2002). The SOP is selected from the PNC by lateral inhibition in a manner akin to the R8

cell. In this case, the cell expressing highest level of proneural activators encoded by the *Achaete-Scute* gene complex in response to pre-pattern factors is destined to become the SOP and in turn initiates lateral inhibition via the E(spl) repressors in the adjacent cells of the PNC by activating Notch (Artavanis-Tsakonas et al., 1999; Castro et al., 2005; Giebel and Campos-Ortega, 1997). The events after SOP selection are however different from those following R8 specification. The SOP undergoes two asymmetric cell divisions to generate the four distinct cell types characteristic of the bristle. The SOP at first forms the pl neuroblast, which then divides asymmetrically to give rise to the pIIa and pIIb cells. Asymmetric division of the pIIa cells generates external socket and shaft cells, which form the external structures of the bristle (Fig. 5). The pIIb cell, on the other hand, divides asymmetrically to give rise to a glial cell and the third order precursor called the pIIIb cell. Asymmetric division of pIIIb cell gives rise to the two internal cells of the bristle, the neuron and the sheath cell (Fig. 5).

A body of genetic evidence supports the reiterated roles of Notch signaling in bristle development. For example, loss of Notch function prior to SOP selection leads to the specification of extra SOPs from each PNC. In adult flies, each of these extra SOPs goes on to form a complete bristle, which manifest as ectopic and closely spaced bristles (Campos-Ortega, 1998; Skeath and Carroll, 1991). On the other hand if Notch function is lost after SOP selection, the stoichiometric sister cell fates become skewed resulting in split bristles or missing bristles (Bray, 1997; Campos-Ortega, 1997). A split bristle reflects a defect in the asymmetric division of the pIIa cell, which transforms a socket cell into a shaft cell. On the other hand, a missing bristle can be a manifestation of either the loss of SOP, itself, or a defect in the asymmetric division of SOP. A defect in the asymmetric division gives rise to two pIIb cells, which result in the specification of two sets of neurons and sheath cells. Because the shaft and socket cells, the external components of the sense organ are missing, this manifests as a missing bristle.

E(spl)-C complex and bHLH repressors

E(spl)-bHLH repressors are the terminal effectors of the Notch inhibitory pathway during eye and bristle development. These proteins repress Ato/ASC during the time of lateral inhibition and restrict neural competency to a single cell of the PNC. The E(spl) complex (*E(Spl)C*), encompasses ~60 Kb region on the 3rd chromosome and encodes 7 structurally similar bHLH proteins (Mγ, Mδ, Mβ, M3, M5, M7 and M8) (Fig. 6), four Bearded family member (BFM) proteins (Mα, M6, M4 and M2), one protease inhibitor, kazal-m1 and the distal, non-bHLH protein Gro (Delidakis and Artavanis-Tsakonas, 1991; Lai et al., 2000; Wurmbach et al., 1999). Gro protein is known to physically interact with the E(spl)-bHLH repressors and is needed for their function (Paroush et al., 1994). Recent studies have shown that the BFM proteins function as inhibitors of Neuralized, an E3 ubiquitin ligase, which is necessary for ubiquitinylation and subsequent endocytosis of the Notch ligands, Delta and Serrate (Bardin and Schweisguth, 2006; De Renzis et al., 2006). This step is necessary for ligand activation.

The E(spl)-locus is conserved across 12 *Drosophila* species and reveals remarkable molecular synteny with respect to both the number of homologs and their position relative to one another (Fig. 6). Given estimates of evolutionary distances between these 12 species (Beverly and Wilson, 1984), it appears that the *E(Spl)C* locus has remained largely unchanged for over 50 myr. In addition, the conservation of consensus sites for several protein kinases in specific members presents a unique opportunity to better understand how these proteins are regulated during development (see below). Initial studies revealed that over-expression of any E(spl) protein elicited loss of bristles and the SOPs (Giebel and Campos-Ortega, 1997; Nakao and Campos-Ortega, 1996). Consequently, these proteins have been considered to be functionally redundant, a view that also extends to their mammalian homologues, the HES proteins. It has been argued that 'redundancy' is an

oversimplification, as the order of the seven E(spl) genes and their direction of transcription is conserved over ~60 myr of Drosophila evolution (Maier et al., 1993).

Over the years, several studies have sought to identify the functional domains and the mechanism of neural repression by E(spl) proteins. All E(spl) bHLH proteins have in order from the N-terminus, a basic domain (DNA binding), an HLH domain (dimerization), a second HLH region, the Orange domain (interaction with Ato/ASC) and a C-terminal WRPW motif (Gro-binding) (Fig. 7). While these domains are highly conserved between individual E(spl) members, their length and/or sequence heterogeneity is largely confined to the C-terminal domain (CtD), the region between Orange and WRPW. This region was therefore thought to not be important for repressor activity, but studies conducted in our lab are revealing that the CtD plays an important role in regulating repression *in vivo*. Specifically it was shown that the CtD of M5, M7 and M8 harbors a highly conserved site for phosphorylation by the kinase CK2 (Fig. 7), one that mediates both E(spl)-CK2 interactions and results in their phosphorylation. These studies thus raised the possibility that these three E(spl) members are regulated by post-transcriptional modification (Trott et al., 2001).

Although much time has been spent investigating the mechanism of neural repression by E(spl) members, no universal mode of action has emerged. It was initially proposed that E(spl) proteins bind to specific DNA elements called the N-box sequence in target genes, recruit Gro and elicit their repression (Tietze et al., 1992; Van Doren et al., 1994). However, it was subsequently shown that deletion of N-box from ASC enhancers failed to impair repression *in vivo*. Moreover, the enhancers of *Ato*, a gene vital for eye development and whose expression pattern is refined by E(spl)-M8, lacks any credible N-box like sequence, suggesting alternative mechanisms underlie repression by these proteins. A more recent study proposed that E(spl) proteins directly interact with enhancer-bound ASC/Atonal proteins and regulate repression via this protein-tether mechanism

(Alifragis et al., 1997; Giagtzoglou et al., 2003; Gigliani et al., 1996). These interactions are thought to involve the Orange domain of E(spl) proteins, a proposal validated by biochemical and yeast-two hybrid assays, where E(spl) members have been shown to (physically) interact with ASC members, Atonal and their co-activator Daughterless (Dawson et al., 1995; Giebel and Campos-Ortega, 1997; Gigliani et al., 1996).

Role of E(spl) members in eye and bristle development

Initial studies involving SOP and bristle development have led to the notion that transcription and accumulation of E(spl) proteins, was by itself, sufficient for neural repression (Giebel and Campos-Ortega, 1997; Nakao and Campos-Ortega, 1996). In contrast, studies in the eye development rendered different results. In the developing eye, the loss of E(spl) did in fact compromise lateral inhibition and result in excess R8 specification, but ectopic expression of M5, M7 and M8 did not elicit a dominant loss of R8 cells (Ligoxygakis et al., 1999). The inactivity of M5 and M7 may reflect the fact that these two members of the E(spl) family are not expressed at this stage of eye development and are thus dispensable for R8 specification and/or patterning (Cooper et al., 2000). However, endogenous M8 is expressed in the MF during R8 specification and its mutation, the *E(spl)D* allele, has also been shown to severely affect R8 patterning and eye formation (see below). Based on these distinct developmental outcomes it was suggested that M5 and M7 must be qualitatively different than M8, a contention, which remained largely unresolved.

The dominant allele *E(spl)D* was first identified in 1956 based on its ability to impair eye formation when combined with the split allele of Notch (*N^{spl}*) (Tietze et al., 1992; Welshons, 1956, 1965). It was in fact the 'enhancement' of the split eye defect of the Notch allele, that led to the nomenclature as 'E(spl)'. *E(spl)D* codes for a truncated protein referred to as M8*, which lacks 56 residues of the CtD. Despite not binding to Gro as it lacks the WRPW motif, M8* displays exacerbated repression of Atonal, thereby blocking R8

birth and eye development. Because M8* lacks the WRPW motif and hence has no ability to bind its co-repressor, it has been suggested that E(spl)D is a 'Gro-independent hypermorph' (Nagel et al., 1999). Surprisingly, such a developmental defect could not be recapitulated simply by removal of the WRPW motif from a full-length M8. This paradox was addressed by the late Dr. Campos-Ortega, who presciently proposed that the CtD of M8 may have a regulatory influence on E(spl)-M8 activity. However, the nature of this regulation remained unknown until the finding that this region between Orange domain and WRPW motif is a target for phosphorylation (Karandikar et al., 2004). It was then that the biochemical mechanism of repression by E(spl)-M8 began to become more clear.

Regulation by E(spl) repressors is phosphorylation dependent

A role for CK2 emerged from the cloning of *m7* transcript based upon its interaction with CK2 in multiple (yeast) two-hybrid screens. Follow up studies revealed that CK2 interacted with only three of the seven E(spl) proteins, M7, M8 and M5. This interaction was mapped to the CtD region, which uncovered a highly conserved consensus for CK2 (Trott et al., 2001). This motif in the case of E(spl)-M8 is S¹⁵⁹DCD (Ser-Asp-Cys-Asp). Subsequent to these studies, it has been found that HES6, the mammalian homolog of E(spl)-M8, harbors a similar phosphorylation motif that is also modified by CK2 in vitro (Gratton et al., 2003b). These results raise the possibility that E(spl) phosphorylation by CK2 is a key regulatory step in Notch signaling during lateral inhibition.

Follow-up studies investigated the role of CK2 in repression by M8, using variants that replace the CK2-phospho acceptor Ser¹⁵⁹ with Asp or Ala. The Ala variant (M8S¹⁵⁹A) renders the protein non-phosphorylatable whereas the Asp modification (M8S¹⁵⁹D) mimics the hyper-phosphorylated state. Overexpression of wild type M8 led to loss of interommatidial bristles (IOBs) and the macrochaetes (MCs) but failed to elicit any ommatidial defects. Also, no eye defects were seen with the non-phosphorylatable isoform,

M8-S¹⁵⁹A. However, overexpression of M8-S¹⁵⁹D elicited strong loss of R8s and a severe reduction of the eye (Karandikar et al., 2004). The R8/eye defects of M8SD are virtually identical to those of M8* (Nagel et al., 1999) which lacks the CtD and its resident CK2 site. Furthermore, yeast two-hybrid assays reveal that M8* and M8-S¹⁵⁹D interact with Ato with equal strength, whereas M8 or M8-S¹⁵⁹A do so only weakly. It was thus proposed that the CtD region likely autoinhibits the Orange domain, previously implicated in Ato binding (Jafar-Nejad et al., 2003). Phosphorylation displaces this '*cis*' interaction permitting repression (Fig. 7). Consequently, the truncation of CtD in M8* removes this regulatory region, thereby bypassing autoinhibition and resulting in a protein that constitutively binds to Ato. If so, it would suggest that CK2 regulates the lateral inhibition mediated by M8. Consistent with these findings, targeted knockdown of CK2 elicits supernumerary R8s from the IGs in the developing retina, and to supernumerary MCs and SOPs in the bristle lineage (Bose et al., 2006). These results reveal a key role for CK2 in the control of E(spl) activity during Notch-mediated lateral inhibition, and that this kinase regulates E(spl)/Notch functions in broader contexts. In a similar manner, CK2 modifies HES6 and this post-translational modification (PTM) is essential for the formation of a HES6-HES1 complex. Thus the role of CK2 in promoting protein-protein interactions between two classes of bHLH proteins appears to be highly conserved. However, the developmental significance of the HES6 phosphorylation and the regulated formation of HES6+HES1 dimer in humans remains unexplored.

The expression of M8 or M8SD in the bristle lineage elicited a loss of MCs with equal severity, a stark contrast with respect to the results seen in the eye (Karandikar et al., 2004). The difference in results between eye and bristle led to the suggestion that role of CK2 might be dispensable in ASC antagonism during bristle development, but that it plays a critical role in eye development. It is possible that the absence of a role for CK2 in the bristle formation reflects a limit phenotype, a function that is not discernible by the approach

of ectopically expressed proteins.

The 'autoinhibition' Paradox

In line with the autoinhibition model, one would expect that both M8SD (phosphomimetic variant) and M8* (lacking the CtD) should be constitutively active repressors of Ato and should enable activity to be evaluated at three critical stages of R8 birth. If anything, their repressive activity should be maximal at stage-1, where Ato levels are the lowest, as compared to that at stage-2/3 of the MF, where Ato levels are at the highest and have achieved a 'threshold' sufficient to drive birth of the R8s. However, contrary to this prediction, the dominant eye defects of M8* and M8SD have been found to be MF stage-specific. It appears that the dominant eye defects of M8SD are stage-2/3 specific where lateral inhibition refines R8s from PNCs. In contrast, M8* elicits a severely reduced eye only at stage-1 of the MF, but fails to exhibit any activity at stage-2/3. If phosphorylation by CK2 was necessary and sufficient to overcome the autoinhibited state, M8SD should have also elicited loss of R8s/eye upon expression at stage-1 in a manner akin to M8*. The inactivity of M8SD at stage-1 therefore raises the possibility that, while important, CK2 is insufficient to activate M8 on its own. If so, are other regulatory enzymes involved?

The possibility of M8 undergoing secondary modifications was hypothesized given that the P-domain conserves a number of Ser residues in addition to that which is modified by CK2 (Ser 159). This clustering of Ser residues within the CtD is not only conserved in *Drosophila* M5/7, but also in murine and human HES6. In the case of HES6, secondary phosphorylation by MAPK, an effector of Epidermal Growth Factor Receptor (EGFR) signaling, has been demonstrated in cell lines, but how this regulates HES6 activity during development has not been reported. Interestingly, upstream of the CK2 site in *Drosophila* M8 lies a PxSP motif, which meets the strict consensus for modification by MAPK. Although

the developmental consequences of phosphorylation of HES6 by MAPK are unknown, the possibility arises that multisite phosphorylation by CK2 and MAPK could be essential for regulation of repression by Drosophila E(Spl) proteins. If M8 activity were to require both CK2 and MAPK, it would establish that EGFR signaling potentiates Notch pathway activity and that these two signaling pathways do not always act in an antagonistic manner, and provide important insights into the complex roles of EGFR signaling during eye development (see below).

Protein Kinase CK2

CK2 is a conserved Ser/Thr protein kinase present ubiquitously in all eukaryotes. It is a highly conserved kinase that plays critical roles in diverse cellular and biochemical events such as gene expression, cell cycle progression, cell signaling, DNA replication and repair, transcription, translation and apoptosis (as reviewed in (Meggio and Pinna, 2003)). The kinase was first isolated and identified from rat liver extracts in the laboratory of Eugene Kennedy (Burnett and Kennedy, 1954). The protein was aptly named 'protein phosphokinase' for its ability to catalyze the addition of phosphate (from ATP) to Ser/Thr residues on the protein casein. However, casein is not a natural (physiological) target of CK2 and thus no biological relevance could be established behind this phosphorylation effect. In 1955, seminal work from Edmond Fischer and Edwin Krebs' lab demonstrated that phosphorylation is an important biochemical step for regulating enzyme activity. Specifically, they showed that glycogen phosphorylase is activated by cAMP-dependent protein kinase (PKA) (Fischer and Krebs, 1955). This discovery has been the foundation of studies identifying and characterizing protein kinases present in biological systems, and efforts to analyze their roles in regulating diverse cellular functions such as cell division, DNA replication, transcription and translation, animal development, etc.

During the purification process of Casein Kinase in Jolinda Traugh's laboratory, two

forms of the enzyme were identified based on their order of elution in column chromatography and consequently these two activities were named Casein Kinase I and II (Hathaway and Traugh, 1979). Following purification, antibodies specific for mammalian CK2 were generated which demonstrated cross-reactivity with homologous enzymes from *Drosophila* and worms (Dahmus et al., 1984). Furthermore, it was shown that the CK2 α subunit from *Drosophila*, human and worms can functionally substitute for one another (Bidwai et al., 1992; Padmanabha et al., 1990). Together, these observations indicate that CK2 α is universally conserved in both structure and function in all eukaryotes.

Biochemistry of CK2

The CK2 holoenzyme is a hetero-tetramer that is composed of two catalytic (α) subunits and two regulatory (β) subunits (Glover et al., 1983; Padmanabha and Glover, 1987). The α -subunit displays the catalytic activity and can utilize either ATP or GTP as a substrate (Glover et al., 1983). The catalytic activity of monomeric CK2 α is stimulated by the regulatory β -subunit, which modulates substrate recognition and provides stability to the holoenzyme (Cochet and Chambaz, 1983). The mammalian enzyme contains two isoforms of the catalytic subunit (α and α') that are encoded by two distinct genes whereas a single gene specifies the regulatory β -subunit.

When compared to other protein kinases, CK2 can utilize either ATP or GTP as phosphoryl donor to phosphorylate Ser/Thr residues (Dahmus et al., 1984; Glover et al., 1983; Hathaway et al., 1980). CK2 preferentially targets acidic substrates and recognizes a unique consensus sequence at its target site. This consensus sequence is S/T-D/E-x-D/E, in which the acidic residues at positions n+1 and n+3 are rate limiting for phosphorylation (Kuenzel and Krebs, 1985; Kuenzel et al., 1987). Additional acidic residues at the N- and C-terminus of this microdomain further enhance phosphorylation activity of CK2 towards the target protein. The effect of the acidic residues in the microdomain can be biochemically

mimicked by pSer/pThr, which generates hierarchical sites thus increasing the likelihood of progressive phosphorylation by CK2. The most extreme example of hierarchical modification is Nopp140, a protein that is progressively modified by CK2 at 72 sites (Meier and Blobel, 1992).

CK2 and its role in development

Since its discovery in 1953, a range of targets for CK2 has been identified by genetic, biochemical and computational studies. The kinase has been thought to play an essential role in different branches of cell biology, which includes cell-cycle control and cell proliferation, circadian rhythmicity, cell signaling and animal development and even in learning and memory. Its role as a cell cycle regulator was first observed when the stimulation of mammalian cells in culture with the mitogen Epidermal Growth Factor (EGF) elicited oscillations in the levels and activity of CK2 (Sommercorn et al., 1987). Moreover, a synchronous oscillation in CK2 activity was seen with G1 to S and G2 to M phase transitions in the mammalian cell cycle, results since corroborated by the isolation and analysis of temperature-sensitive alleles of yeast CK2 (Hanna et al., 1995; Rethinaswamy et al., 1998). In another study, it was shown that deletion of the genes encoding the catalytic subunit of yeast CK2 (CKA1 and CKA2) resulted in a cell death, and cells depleted of CK2 displayed a 'pseudomycelial' or 'Shmoo' morphology, a classic cell division cycle (CDC) phenotype reflecting the uncoupling of cell growth from cell division in budding yeast (Padmanabha et al., 1990). The most unique role of CK2 is perhaps displayed during Theileriosis, a B- and T-cell lymphoproliferative disorder in cattle. It was the pioneering work of Dr. Ole-Moi Yoi, a veterinarian in Kenya, which displayed that cattle exhibiting the symptoms of this disease invariably harbored the parasitic protozoa, *Theileria parva*, in their circulatory system. His work subsequently demonstrated that this parasite preferentially infects B- and T-cells and overexpresses a unique secretory form of CK2, which accumulates in the cytosol of the host

cell (ole-MoiYoi, 1995; ole-MoiYoi et al., 1993). This parasite-derived CK2, which bears striking similarity with the normal (mammalian) variant of the kinase, likely deregulates the normal control of the cell cycle, which then elicits the development of lymphomas.

In addition, CK2 has been shown to associate with mitotic spindles and the centrosomal apparatus (Yu et al., 1991). It also interacts with vital regulators of cell division and replication checkpoint controls and targets a number of proto-oncogene-derived proteins such as Myc, Myb, Fos and Jun (Luscher et al., 1989; Winkler et al., 2000). Besides its role in development of tumors, CK2 has been closely linked to metastasis, for which substantial efforts are underway to design specific inhibitors of this enzyme and suppress the detrimental effects.

Given the vital role of CK2 in diverse biological processes, significant efforts have been made to characterize the large number of targets of CK2. High throughput techniques and availability of genomic sequences have made it possible to identify a large number of targets for CK2. These interactions include proteins involved in various types of cellular and biological functions such as DNA replication, transcription and translation, cell polarity and development, cytoskeleton architecture and cancer (reviewed in (Meggio and Pinna, 2003)). Therefore, the loss of CK2 elicits cell lethality in all unicellular and metazoan organisms.

Drosophila CK2

Drosophila CK2 was first purified from embryos and similarly to its mammalian counterpart was shown to be a tetramer, composed of two α subunits and two β subunits ($\alpha_2\beta_2$ holoenzyme). A single *CK2 α* gene located on the 3rd chromosome encodes the catalytic subunit, whereas the regulatory subunit is encoded by *CK2 β* gene on the X chromosome (Bidwai, 2000).

To date, two spontaneous mutations of the *CK2 α* gene has been identified based on their ability to elicit defects in the circadian clock. These are known as *Timekeeper* (Tik),

and its partial revertant, called *Tik^R* (Lin et al., 2002). *Tik* harbors two substitutions, M¹⁶¹K and E¹⁶⁵D. The M¹⁶¹K substitution is located within the ATP-binding pocket, thus interfering with the enzymatic activity of CK2 (Rasmussen et al., 2005). Accordingly, *Tik* is homozygous lethal and the lethality is manifested at first instar larval stage. The second substitution, E¹⁶⁵D lies in a highly conserved motif, H E¹⁶⁵NRKL, which mediates physical interaction between human CK2 α and protein phosphatase PP2A (Heriche et al., 1997). Although thought to be a silent mutation at first because it involved a conservative replacement, the importance of E¹⁶⁵D mutation was soon realized as it impairs downregulation of phosphatase activity. Thus, *Tik* has been aptly suggested to be a 'double hit', inactivating CK2 activity on one hand and enhancing PP2A activity on the other hand (Kunttas-Tatli et al., 2009).

Tik^R was identified as a spontaneous revertant allele, based on its ability to partially compensate for the circadian clock defects in *Tik/+* animals. In addition to the two mutations already present in the *Tik* allele, *Tik^R* harbors an R²⁴²G (Arg to Gly) substitution and a deletion of seven internal amino acids (234-240). Consequently, *Tik^R* is also homozygous lethal, manifesting its lethality at first instar stage (Lin et al., 2002). Studies indicate that *Tik^R* is insoluble, which points to defects in protein folding mechanism, a defect not seen with *Tik*. Moreover, unlike wild type CK2 α or mutant *Tik*, the *Tik^R* protein does not interact with CK2 β , an interaction, that is essential for tetrameric holoenzyme assembly (Kunttas-Tatli et al., 2009). These observations led to the view that *Tik* integrates into and 'poisons' the endogenous holoenzyme, thus compromising its activity. In contrast, since *Tik^R* is unable to assemble into the holoenzyme, it shows a revertant behavior in context to the circadian clock where halve dosage of CK2 (*Tik^R/+*) may still be sufficient to maintain near-normal functionality of circadian rhythms. Apart from clock defects, neither *Tik* nor *Tik^R*, display any overt developmental abnormalities in heterozygous condition, indicating that the

residual levels of CK2 are sufficient for normal developmental functions of the cells/tissues.

Recently, a third allele of *CK2 α* , *CK2^{MB0047}* has been described that harbors a transposable *minos* element in the 5'UTR (Bellen et al., 2004). Unlike, Tik or TikR, *CK2^{MB0047}* homozygotes manages to complete all three larval stages, but manifest in lethality at the pupal stage. This indicates that *CK2^{MB0047}* is a hypomorph and consistent with its characteristic fails to elicit any eye or bristle defects in heterozygous state.

An allele of *CK2 β* (*andante*) also displays clock defects. The mutant protein fails to form a stable holoenzyme in vivo. However, in vitro analysis with the human variant of *andante* challenges this idea, and the mechanism underlying the clock defect in *Drosophila* remains unresolved (Rasmussen et al., 2005). Homozygous *andante* flies are viable, indicating that it is a hypomorphic allele (Akten et al., 2003).

The Epidermal Growth Factor Receptor (EGFR) Pathway

The Epidermal Growth Factor Receptor (EGFR) Pathway is a conserved pathway that regulates diverse processes critical for metazoan development, ranging from cell proliferation and survival to cell-fate specification to differentiation (Doroquez and Rebay, 2006). Reflecting this importance, several efforts have been made to understand the signal transduction and the underlying mechanism of each member involved in this pathway (Yarden, 2001). The pathway has been studied extensively in *Drosophila*, *C.elegans* and mammalian cell lines and several computational models have been analyzed to understand its dynamics (Schoeberl et al., 2002; Voas and Rebay, 2003; Wassarman et al., 1995).

EGFR Receptor and Ligands

EGFR is a transmembrane receptor belonging to the family of receptor tyrosine kinases. These receptors are anchored in the cell membrane and contain an extracellular ligand-binding domain, a short hydrophobic trans-membrane region, and an intra-cytoplasmic tyrosine kinase domain. In *Drosophila* and *C.elegans*, the receptor is coded by

a single gene namely *Drosophila* EGF receptor (DER) and *let-23* respectively. Mammalian EGFR consists of four members, which include EGFR/ErbB1/HER1, ErbB2/HER2/Neu, ErbB3/HER3 and ErbB4/HER4 (Yarden, 2001).

In the *Drosophila* system there are four activating ligands- Spi (Spi), Keren (Krn), Gurken (Grk) and Vein (Vn) and one secreted inhibitor Argos (Aos) (Neuman-Silberberg and Schupbach, 1993; Reich and Shilo, 2002; Rutledge et al., 1992; Schnepp et al., 1996). Spi, Krn and Grk are all synthesized as membrane-bound precursors and are subsequently cleaved by a Rhomboid family protease to be secreted (Urban et al., 2002). The membrane protein Star plays a role in the EGFR pathway by trafficking the ligand precursors from the ER to the Golgi where they can be cleaved by Rhomboid for secretion (Lee et al., 2001). Spi is the principle ligand activating the DER pathway in the majority of developmental contexts, including *Drosophila* eye morphogenesis, while Krn and Grk are less frequently involved (Freeman, 1994). Vn belongs to the Neuregulin family and is utilized in a number of tissues such as in the wing, muscle attachment sites and distal leg regions (Campbell, 2002; Yarnitzky et al., 1997). The inhibitory ligand, Aos is unique to *Drosophila* so far, as no mammalian/worm homologue has been identified. DER signaling leads to enhanced *aos* expression, which suggests that it plays a negative feedback loop role, thus regulating EGFR pathway output (Wasserman et al., 2000). Given the import of this negative feedback loop, it remains enigmatic why a mammalian counterpart has remained unidentified.

In mammals the ligands that regulate ErbB receptors can be separated into two main groups. 'EGF agonists' activate EGFR and Neuregulins that bind ErbB3 and ErbB4 (Yarden and Sliwkowski, 2001). There are no known ligands for ErbB 2 and it is thought to play a role in EGFR pathway by forming heterodimers with other ligand-activated ErbB family members. In *C.elegans*, the ligand for EGFR is Lin-23. It is a soluble TGF- α -like ligand whose roles have been extensively studied during vulva development.

EGFR pathway activation

In the absence of ligand, EGFR is monomeric and the cytoplasmic tail shows no kinase activity. When ligand binds to the extracellular domain of the receptor, it induces receptor homo- or hetero-dimerization, which leads to subsequent activation of the intrinsic tyrosine kinase domain. The cytoplasmic tail of the receptor undergoes 'trans-phosphorylation' where one receptor monomer phosphorylates the Tyrosine residues of the other binding partner. The phosphorylated residues of the dimerized receptor serve as docking sites for downstream signaling proteins. The activated receptors also mediate signals by phosphorylating target proteins. EGFR hetero-dimerization is a unique feature of the mammalian system (Schlessinger, 2000). In contrast, *Drosophila* and *C. elegans* exhibit only homodimerization due to the presence of only one isoform of EGFR. Receptor heterodimerization has been attributed to diversity and specificity of EGFR function and to signal output.

MAPK pathway

Among all the different signaling pathways activated by EGFR, the MAPK pathway is a widely studied and critically important signal transduction mechanism for regulating cell proliferation and cell survival. The pathway works via a three-tier phosphorylation cascade in which the terminal (effector) kinase, MAPK, is activated upon phosphorylation by mitogen activated protein kinase (MAPKK), which in turn is activated upon phosphorylation by MAPKKK. Activated MAPK either phosphorylates proteins in the cytoplasm or in the nucleus, targeting various transcription factors and thus regulating transcription (Reiser et al., 1999). To date, six different MAPKs have been identified in mammals; extracellular regulated kinases (Erks 1/2, Erks 3/4, Erks 5, Erks 7/8), Jun N-terminal kinase (JNK) 1/2/3 and p38 isoforms α / β / γ (Erk 6) and δ (Krens et al., 2006; Kyriakis and Avruch, 2001; Schaeffer and Weber, 1999). *Drosophila* expresses all three subgroups of the MAPK;

Rolled (Rl), Basket (Bsk) and p38a/b. Rolled and Basket are Erk and JNK homologs, respectively, whereas p38a/b are homologs of p38. EGFR signaling in *Drosophila*

The EGFR pathway in *Drosophila* begins with Spi binding to the extracellular domain of DER. Ligand binding activates DER, which undergoes autophosphorylation and in turn activates a downstream GTPase called Ras (Seeger and Krebs, 1995). Activated Ras then activates Raf1 (MAPKKK), which then phosphorylates and activates the dual specificity kinase MEK (MAPKK). The kinase MEK then phosphorylates MAPK (ERK) at Tyr and Thr residues within the activation loop (Zhang et al., 1995). Dual-phosphorylated MAPK (dpERK) targets both cytoplasmic and nuclear proteins, phosphorylating at Ser/Thr/Tyr residues (Reiser et al., 1999). In *Drosophila*, the nuclear targets of dpERK include ETS transcription factors Pointed (Pnt) and Anterior open (Aop), also referred to as Yan (O'Neill et al., 1994; Rebay and Rubin, 1995). Pnt, a transcriptional activator and Yan, a repressor, both harbor an ETS-DNA binding domain, by virtue of which they compete for the regulatory regions of the downstream transcriptional targets. Upon DER signaling, dpERK phosphorylates Yan, which then undergoes nuclear export and subsequent degradation. dpERK simultaneously phosphorylates Pnt converting it into a strong activator, which then activates transcriptional targets previously repressed by Yan (Brunner et al., 1994; O'Neill et al., 1994). This dual regulation thus confers bimodality of EGFR signaling in flies.

Negative regulators of the EGFR pathway also play a key role in fine-tuning the complex signaling profile of this developmental pathway. In *Drosophila*, high level EGFR activity upregulates transcription of the inhibitory ligand Aos (Golembo et al., 1996). Aos inhibits EGFR signaling without directly interacting with the receptor. Instead, Aos binds to the Spi, thus sequestering it. The Spi-Aos complex does not bind to the receptor and as such a low steady state level of EGFR signaling is maintained in the cell (Klein et al., 2004).

Role of EGFR in Drosophila eye and bristle development

From specification of the MF, to the specification of the different photoreceptors and their roles in ommatidial spacing, EGFR plays a critical role either working cooperatively or antagonistically with Notch.

The EGFR pathway has been implicated in the initiation of the MF, upstream of Hh and Dpp signals. It has been proposed that the EGFR pathway plays a role in both furrow birth (formation of the posterior margin) and furrow reincarnation (formation of MF along the lateral margin) during Drosophila eye development (Kumar and Moses, 2001). A study conducted by Kumar and Moses suggests that during birth of the MF, the EGFR pathway signals to Notch which in turn signals to Hh (Hedgehog). EGFR-mediated expression of Hh induces photoreceptor differentiation, which, in turn, induces elevated EGFR signaling and activation of the downstream effector Pnt. Activated Pnt confers regulation on Hh, thus establishing a positive feedback loop which facilitates continued forward progression of the MF (Rogers et al., 2005). During the furrow reincarnation step EGFR signals to Notch which, in turn, activates the Dpp pathway (Kumar and Moses, 2001). Dpp is expressed along the posterior and lateral margins of the developing retina, its ectopic expression leads to the formation of precocious furrows along the anterior margins (Chanut and Heberlein, 1997).

It has been suggested that EGFR positively regulates expression of the homeo domain protein Rough (ro) in the MF. Ro is expressed in the PNC, which induces Ato expression in all but R8 cells, consequently selecting a single R8 cell. Thus EGFR indirectly controls the singling out process of R8 cells by maintaining Ro expression in the PNC (Dominguez et al., 1998).

The EGFR pathway plays an important role in macrochaete morphogenesis in Drosophila, and accordingly hypomorphic mutations of the *Egfr* gene have been shown to elicit the loss of several notum macrochaete with varying frequencies (Clifford and

Schupbach, 1989). In mosaic clones of hypomorphic *Egfr* alleles, both the absence and the duplications of macrochaetes have been observed (Diaz-Benjumea and Garcia-Bellido, 1990). The overexpression of the inhibitor of EGFR pathway, Aos, in the PNC results in the loss of essentially all macrochaetes. It has been suggested that only a subset of bristles require DER signaling. The notum macrochaetes, whose positions are invariant, are sensitive to lowered dosage of DER signaling and fail to be specified in a precise manner, the microchaetes are much more resistant to the lowering of the DER signaling (Culi et al., 2001). This is perhaps an outcome of the invariant nature of the microchaetes, which are organized in density patterns instead of being specified at fixed positions in the notum.

Suspected role of EGFR signaling during R8 specification in *Drosophila*

While there is abundance of evidence for the contribution of MAPK to retinal determination, the role of the EGFR pathway in R8 selection is unclear. Analysis of the EGFR allele *Ellipse* (*Elp*) revealed loss of intermediate group or R8 cells, suggesting a role of *Egfr* in R8 specification (Lesokhin et al., 1999; Yang and Baker, 2001). *Elp* also exacerbated the effects of the Notch allele, *N^{sp1}* (Baker and Rubin, 1992), which by itself exhibits a gain-of-function behavior affecting R8 selection. Moreover, ectopic activation of EGFR pathway by several mechanisms was reported to enhance Ato expression and specify ectopic R8s (Yang and Baker, 2001). It has also been suggested that the transient expression of the dominant-negative EGFR, DER^{DN}, blocks R8 specification and proneural cluster formation. In addition, ectopic expression of Aos leads to the loss of Ato expression, which, in turn, results in the loss of and aberrant spacing of R8s (Spencer et al., 1998). Consistently, reducing Aos activity results in the formation of ectopic R8s, although disruption of ommatidial patterning is not very severe in such a case (Freeman et al., 1992; Spencer et al., 1998).

Investigators have detected dp-ERK, an active form of the MAPK, in the cytoplasm,

several hours after EGFR activation (Kumar et al., 1998). This activated MAPK staining was absent in *EGFR^{null}* clones, suggesting that dp-ERK accumulation is a direct downstream effect of EGFR signaling and neither an artifact nor can be attributed to the activation of other RTKs such as Sevenless (Lesokhin et al., 1999). The cytosolic hold on this kinase would lead one to think that its established targets, the ETS family transcription factors Pnt and Yan/Aop, are not the canonical targets of this pathway. Indeed if one looks at data from the laboratory of Nicholas Baker, in clones of Pnt (Yang and Baker, 2003) there is no evidence of defects in R8 spacing, suggesting that in this particular context the EGFR pathway could well be used in a non-canonical manner, where the Egfr effects are not realized within the nucleus. Based on these observations it has been suggested that EGFR may cooperate with the Notch pathway to restrict R8 fate to a single cell within a PNC during lateral inhibition.

Preface to chapters 2-4 of this dissertation

The studies described in Chapter 2 and 3 of this dissertation seek to address the molecular mechanism of repression by M8 during R8 selection and patterning. These studies should determine the contribution of EGFR/MAPK signaling in Ato repression by M8. The proposed role of EGFR in R8 selection has heretofore lacked a molecular target. The characterization of M8 as a mediator of EGFR pathway during R8 specification will address this unresolved question.

In chapter 2, the studies described involve site-specific (Asp/Ala) variants of M8 to reveal the role of CK2 and MAPK in repression of Ato. The findings, based on both gain- and loss-of-function analyses, are consistent with the possibility that the two regulatory inputs, CK2 and MAPK, modulate Notch activity through sequential phosphorylation of the CtD of the M8. Our studies indicate that both modifications of the Ser residues in the P-domain are necessary to relieve the autoinhibited state of M8 and elicit its repressor activity during

neurogenesis. Moreover, studies using spatially controlled expression of M8 variants suggest that CK2 is likely to play a more permissive role, whereas MAPK controls when and where M8 is activated. Such a control mechanism through two kinases may control both the amplitude and duration of active M8 within the MF.

In chapter 3, I more directly evaluate the role of the P-domain in autoinhibition. I have generated deletion constructs, which either lack the CK2 motif, or the MAPK motif or the entire P-domain. The experiments described in this chapter of the dissertation address the important question whether CK2 and MAPK phosphorylation are required purely (solely) at the level of intramolecular protein contact(s) and whether dual modification of these two sites are necessary to restrain repression by M8.

A. Components of Notch Pathway

Component	Drosophila	C.elegans	Mammals
Receptor	Notch	LIN-12 ,GLP-1	Notch-1,2,3,4
Ligand	Delta, Serrate	LAG-2,APX-1	Delta-1,3,4; Jagged-1,2
Nuclear effectors	Su(H)	LAG-1	CBF-1/RBP-Jk
Canonical Target	E(spl)	REF-1	Hes/Hey

B. Structure of Drosophila Notch

Fig. 1: Core components of Notch pathway and Drosophila Notch Receptor.

(A) The core components of Notch pathway as seen in different species. (B) Schematic representation of the domain organizations in Drosophila Notch receptor.

Fig. 2: Schematic representation of Notch signaling pathway.

The primary steps in Notch activation involve glycosylation and cleavage by Furin (S1 cleavage) followed by receptor maturation. When ligand binds to the Notch receptor of an adjacent cell, the receptor undergoes two consecutive proteolytic cleavages, releasing NICD, which translocates to the nucleus and mediates transcription of the E(spl)/HES genes.

Fig. 3: Lateral inhibition during R8 specification in *Drosophila* eye development.

Notch signaling plays a role in lateral inhibition, as seen between adjacent cells in the proneural cluster (PNC). This inhibitory signal is contact-dependent and is mediated by the ligand Delta, which appears on the cell surface of one cell and binds to the Notch proteins on the neighboring cells. The future R8 cell (orange) is shown to express high levels of Delta, which binds to the Notch receptor in the adjacent non-R8 cell (white) and triggers inhibitory Notch signaling. The pathway terminates with expression of *E(spl)* repressors (here M8 is indicated) that along with co-repressor Groucho antagonizes proneural protein Atonal.

Fig. 4: Compound eye of *Drosophila* and mechanism of R8 specification and patterning.

SEM shows the structure of the adult compound eye in *Drosophila*, which is an array of precisely positioned cluster of unit eyes known as the ommatidium. (A) The bold arrow indicates the progression of the morphogenetic furrow (MF, blue vertical stripe) from the posterior to the anterior part of eye disc. (B) Eye disc showing the expression pattern of the proneural protein Atonal, which is expressed in a broad stripe ahead of the MF, but posteriorly resolves into single cells that become R8's. (C) A schematic representation of the MF showing the biphasic role of Notch during R8 specification. Inset below represents the sequential recruitment of the secondary photoreceptors following R8 specification. (Panels A and B adapted from (Frankfort and Mardon, 2002))

Fig. 5: Bristle morphogenesis in *Drosophila*.

(A) The position of the Macrochaetes (MCs - yellow asterisk) and the microchaetes (mcs - black arrow) on the nota of the fly body. (B) Position of the interommatidial bristles (IOBs - yellow asterisk) on the eye of fruit fly. (C) Bristle morphogenesis requires Notch mediated lateral inhibition during SOP selection followed by asymmetric division of the SOP for lineage specification. Inset shows the four different cell types that constitute the structure of a bristle.

A: E(spl)C organization

B: Molecular synteny of E(spl) locus

Fig. 6: E(spl) C locus and its molecular synteny across 12 Drosophila species.

(A) Schematic representation of the genomic organization of E(spl) locus. It encodes for seven E(spl) repressors and the co-repressor Groucho. E(spl)- mδ, mγ and m8 are expressed in the MF (green boxes). Proteins encoded by E(spl)- mγ, m5, m7 and m8 have conserved CK2 sites (highlighted in red). (B) Molecular synteny of E(spl) locus across 12 Drosophila species. Note (red arrow pointing at centromere) that there is a complete inversion of the locus for six Drosophila species, although the order of the transcription units remains intact (adapted from Majot et al., 2015)).

A: Structure of M8

B: Repression by M8

Fig. 7: Structure of M8 and autoinhibition model of M8 regulation.

(A) Schematic representation of the functional domains of E(spl)M8. Conserved Ser residues in the phosphorylation domain (P-domain) of the CtD in M8, M5, M7 and human and murine HES6 proteins. The yellow boxes represent the MAPK and CK2 motifs. Additional conserved Ser residues are also highlighted (red). (B) Autoinhibition via CtD may block dimerization via HLH (blue) or Ato binding via Orange domain. Phosphorylation of M8 by CK2 regulates antagonism of Ato/ASC in N⁺ flies, whereas M8* requires N^{spl} background (adapted from (Kahali et al., 2010b))

A: Structure of CK2

B: Alleles of the α (catalytic) subunit of CK2

Fig. 8: Structure of CK2 holoenzyme and the alleles of Drosophila CK2α subunit.

(A) Schematic representation of the CK2 holoenzyme. It is a tetramer made up of α (catalytic) and β (regulatory) subunits. Ribbon diagram of the human CK2 holoenzyme (as described in (Niefind et al., 2001)). The two catalytic subunits (magenta) assemble on the two regulatory (yellow and blue) subunits. (B) The three alleles of Drosophila CK2α subunit – CK2MB0047, Tik and TikR and their corresponding lesions are indicated.

Fig. 9: Components of the Drosophila EGFR pathway.

Ligand binding activates EGFR receptor on the cell surface, which undergoes autophosphorylation and activates downstream effector molecules. The downstream signaling cascade activates MAPK, which translocates to nucleus and phosphorylates Pointed (Pnt) and Yan.

CHAPTER 2: The conserved MAPK site in the Notch effector E(spl) M8 controls repressor activity in the eye

Abstract

Enhancer of split (*E(spl)*) M8, an effector of *Drosophila* Notch signaling, is activated by protein kinase CK2 and inhibited by the phosphatase PP2A. CK2 targets a region of M8 that conserves additional Ser residues. One of these Ser residues meets the strict consensus for MAPK, a kinase that mediates the effects of Epidermal Growth Factor Receptor (EGFR) signaling. The studies described here reveal an important role for the conserved MAPK site on M8. Our analysis, using Ala/Asp mutations, suggests that together CK2 and MAPK ensure that M8 repression of Ato occurs in a timely manner and at a precise stage (stage-2/3) of R8 birth. M8 repression of Ato is mitigated by halved EGFR dosage, and this effect requires an intact MAPK site. Accordingly, variants of M8 with a phosphomimetic Asp at the MAPK site exhibit earlier (inappropriate) activity against Ato at stage-1, where a positive feedback-loop is needed to raise Ato levels to a threshold sufficient to specify the R8 fate. Using deletion variants, we demonstrate that both kinase sites likely contribute to 'cis'-inhibition of M8. This key regulation by CK2 and MAPK is bypassed by the *E(spl)D* mutation encoding the truncated protein M8*, which inhibits Ato at stage-1 of R8 birth. Finally, we provide evidence that PP2A likely targets the MAPK site, and that regulation of M8 by MAPK appears to be dispensable during bristle development. Together, our studies reveal that multi-site phosphorylation controls timely onset of M8 repressor activity in the eye, but does not appear to play a role in the bristle. The high conservation of the CK2 and MAPK sites in the insect *E(spl)* proteins M7, M5 and My, and their mammalian homologue HES6, suggest that this regulation may enable *E(spl)*/HES proteins to orchestrate repression by distinct tissue-specific mechanisms, and is likely to have broader applicability than previously recognized.

Introduction

The external sensory organs of *Drosophila* include the compound eye and the mechanosensory bristles which are patterned and established during the larval and pupal stages of development. The remarkably precise hexagonal geometry of the compound eye of fruit flies has afforded an ideal system to understand pattern formation during development. The compound eye of *Drosophila* is composed of ~800 light sensing repetitive units (ommatidia) arranged in a hexagonal lattice. Each ommatidium is composed of eight photoreceptors (R1-R8), twelve accessory cells and an interommatidial bristle (IOB).

During the third larval stage (instar), eye morphogenesis initiates at the posterior margin of the eye disc, a monolayer neuro-epithelium that is the progenitor of the retina. Retinal neurogenesis starts with the specification of the R8 photoreceptors in a moving wave of differentiation, called the morphogenetic furrow (MF) (Jarman et al., 1994). While R8 selection occurs within the MF, all secondary photoreceptor recruitment occurs posterior to it (Wolff and Ready, 1991). The temporal nature of the MF thus represents a 48-hour window, encompassing retinal neurogenesis.

R8 specification occurs in the MF and involves biphasic Notch signaling. At stage-1, the leading edge of the MF, Notch induces the expression of the proneural bHLH activator Atonal (Ato) at a low level to form the 'Ato-stripe'. Heterodimers of Ato and Daughterless (Da) then elicit high levels of *ato* transcription. This process, called *ato* auto-activation, generates neural competency in a group of equipotent cells termed proneural clusters (PNCs). At stage-2/3 of the MF, the future R8s of the PNC express high level of ligand Delta (DI), which interact with the Notch (N) receptors of the adjacent cells (Simpson, 1990). Activated N receptors undergo cleavage and release the intracellular domain (N^{icd}), which then translocate to the nucleus. In the nucleus, N^{icd} interacts with Suppressor of Hairless (Su(H)) and elicits transcription of bHLH repressors encoded by the *Enhancer of split*

Complex (Eslp(C) (Bailey and Posakony, 1995; Delidakis and Artavanis-Tsakonas, 1991; Lecourtois and Schweisguth, 1995). The E(spl) proteins, in a complex with Gro, refines Ato expression to a single cell of the PNC (Ligoxygakis et al., 1998; Paroush et al., 1994). This phase of inhibitory Notch signaling where R8 cell fate is restricted to one cell in the pre-cluster is known as 'lateral inhibition'. By stage-4, single patterned R8s emerge and differentiate as the R8 photoreceptors by sustaining expression of the Ato target gene *senseless (sens)*. The R8s then systematically recruit surrounding uncommitted cells as the R2/R5, R3/R4, R1/R6 cells, concluding with the specification of the R7 cell (Cagan and Ready, 1989).

Given that lateral inhibition is critical for proper structure and patterning of the eye and E(spl) proteins are central to this process, several genetic studies have sought to analyze their modes of action (Giebel and Campos-Ortega, 1997; Knust et al., 1987; Nakao and Campos-Ortega, 1996; Preiss et al., 1988; Ziemer et al., 1988). Although these studies were the first of their kind to establish the role of E(spl) proteins in eye patterning, it has been difficult to identify their exact roles because *Eslp(C)* encodes seven bHLH proteins with similar functional domains. To complicate things further, individual mutations affecting each transcriptional unit have been unavailable, making it impossible to study their individual effects on pattern formation. Initial studies revealed that over-expression of any E(spl) protein elicited loss of bristles and the SOPs (Giebel and Campos-Ortega, 1997; Nakao and Campos-Ortega, 1996). Consequently, these proteins have been considered to be functionally redundant (Cooper et al., 2000), a view that also extends to their mammalian homologues, the HES proteins. It has been argued that 'redundancy' is an oversimplification, as the order of the seven E(spl) genes and their direction of transcription is conserved over ~60 myr of Drosophila evolution (Maier et al., 1993).

Three E(spl) members, M8, M γ , M δ , are expressed in the eye tissue during R8

selection but only ectopic M8 elicited loss of R8s (Ligoxygakis et al., 1998). It therefore, raises the possibility that these three *E(spl)* members are 'qualitatively different', but the underlying nature of this difference remained unresolved.

The central role of M8 during R8 selection is underlined by the R8 and eye defects of the unique dominant allele *E(spl)D* (Welshons, 1956). In the presence of a gain of function Notch allele, *N^{spl}*, the *E(spl)D* allele disrupts retinal histogenesis. It is this interaction that led to the original identification of the *E(spl)* locus. *E(spl)D* encodes a truncated protein called M8*, which lacks 56 C-terminal residues (CtD) (Nagel et al., 1999; Tietze et al., 1992). It was observed that ectopic M8*, but not full length M8, mimics the R8 defects of *E(spl)D*, indicating that the truncation results in a hyperactive protein. The hyperactivity of M8* indicated that the CtD region is likely to regulate repressor activity of M8. Studies in our laboratory, identifying the phosphorylation of M8 at its CtD by protein kinase CK2 provided key insights into the mechanisms underlying hyperactivity of M8* and thus regulation of repression by M8 (Trott et al., 2001).

CK2 phosphorylates M8 at Ser159 in the CtD. Replacement of the CK2 phospho-acceptor (Ser159) with a phosphomimetic Asp residue (M8-S159D) was found to elicit exacerbated loss of R8s and the eye with a potency akin to *E(spl)D* (Kahali et al., 2009a; Karandikar et al., 2004; Nagel et al., 1999). Moreover, M8-S¹⁵⁹D interacts with Ato with a binding strength equal to that of M8*. Consequently it was proposed that the CtD autoinhibits full length M8 (Kahali et al., 2010a). Phosphorylation displaces this intra-molecular interaction permitting repression of Ato. The truncation of M8* would bypass this regulation, implicating CK2 as a regulator of R8 selection through the activation of M8 (Karandikar et al., 2004). Accordingly, loss of CK2 in the imaginal disc of an otherwise wild type flies compromises lateral inhibition and elicits the specification of excess R8s from the IGs (Bose et al., 2006).

The eye defects of the CK2-mimic M8-S159D are specific to stage-2/3 of the MF. If CK2 were to solely control auto-inhibition, the expression of M8-S159D at stage-1, where Ato levels are the lowest, should have also elicited loss of R8 and the eye. The inactivity of M8-S159D at stage-1 raises the possibility that CK2 may be insufficient to activate M8. If so, are other regulatory enzymes involved? This possibility is likely given that the CK2 site in M8 is located in a region termed the P-domain that conserves a number of other Ser residues. The P-domain is conserved in all M8 isoforms through ~60 million years of Drosophila evolution. The Ser residues represent consensus motifs for phosphorylation by MAPK, CK1 and GSK3. The high conservation of Ser residues, in addition to those modified by CK2, raises the possibility that M8 may be regulated by multisite phosphorylation. Although no R8 defects have been described for mutations in CK1 and GSK3, numerous lines of evidence implicate EGFR signaling in R8 selection. This pathway is also known as the Drosophila EGF receptor or DER pathway in Drosophila.

Studies from several groups have shown the presence of active MAPK (diphosphorylated ERK) in IG cells at stage-2/3 of the MF (Kumar et al., 2003; Kumar et al., 1998; Spencer et al., 1998; Yang and Baker, 2003); this staining is lost in the clones of EGFR (Lesokhin et al., 1999) demonstrating that other RTKs such as Sevenless, are not responsible for activating MAPK. Moreover, in DER clones the IGs do not resolve into single R8 and excess R8s are specified (Baonza et al., 2001). An identical effect is seen in eye disc lacking the downstream effectors of the DER pathway, i.e., Ras and Raf (Yang and Baker, 2001). In the canonical DER pathway, activated MAPK targets the nuclear transcription factors Pointed (Pnt) and Yan. Surprisingly, unlike DER clones, extra R8s are not seen in clones lacking *pointed* (Yang and Baker, 2003). In addition, activated MAPK in the IGs is held in the cytosol (Kumar et al., 2003), a location that should prevent its targeting of nuclear-only Pnt or Yan (Shilo, 2003). The possibility remains that the role of DER/MAPK is to target and activate M8 allowing for repression of Ato. In a sense, DER may therefore

control when and where M8 activity manifests.

The studies we describe here indicate the importance of MAPK motif in M8 repressor activity. Site-specific as well as deletion mutants of CK2 and MAPK sites of M8 have been used to demonstrate the role of these two kinase motifs during R8 specification. Our studies indicate that modifications at both sites appear to be necessary for M8 repressor activity and modification at the CK2 site is epistatic to phosphorylation at the MAPK site. If M8 activity were to require both CK2+MAPK, it would establish that EGFR signaling potentiates Notch pathway activity; that these two signaling pathways do not always act in an antagonistic manner, and provide a molecular target for activated MAPK in IG cells.

Results

M8 repressor activity in the developing eye requires multi-site phosphorylation

The birth of patterned R8s in the MF (Fig. 1A) hinges upon timely repression of Ato by E(spl) proteins. The importance of E(spl)-M8 is supported by the severely reduced eye of the *m8* mutation *E(spl)D* (encoding M8*) and that of a variant (M8-S159D) harboring Asp in place of Ser159, the target for protein kinase CK2 (Fig. 1B). The latter studies, which utilized Gal4 drivers with expression at distinct stages of R8 ontogeny (Fig. 1A), reveal that phosphorylation of Ser159 is key to Ato repression (Fig. 1B) (Karandikar et al., 2004). The reduced eye of M8-S159D is seen with *scaGal4* and *109-68Gal4*, whose stage-2/3 expressivity (Fig. 1A) correlates to endogenous *E(spl)* genes. The difference in severity of the reduced eye (Fig. 1B) reflects stronger strength of *scaGal4*, as compared to *109-68Gal4* (Doherty et al., 1997). No reduced eye is seen with wild type M8 (Fig. 1B), a finding previously shown by others (Giebel and Campos-Ortega, 1997; Nagel et al., 1999). It was proposed that CK2 converts 'cis'-inhibited M8 to a state active for binding and repressing

Ato. This regulation is bypassed by the Asp at the CK2 site (M8-S159D), or upon loss of the CtD and the P-domain in M8*, the product of *E(spl)D* (see schematic in Fig. 1B).

Follow-up studies suggest that activation of M8 by CK2 is an oversimplification. First, unlike its reduced eye at stage-2/3, expression of M8-S159D at stage-1 (*h^{H10}Gal4*) is without effect (Fig. 1B). This stage-1 inactivity was unexpected as Ato levels are lowest at this point of R8 birth (Fig. 1A), and should have been most sensitive to repression by (non 'cis'-inhibited) M8-S159D. This inactivity does not reflect weak strength of *h^{H10}Gal4*, as expression of M8* with this Gal4-driver elicits a near complete loss of the eye (Fig. 1B, and see refs). Second, we have recently reported that the reduced eye of M8-S159D is strongly mitigated by increased activity of the phosphatase PP2A (Bose et al., 2014). Because replacement of Ser159 with Asp should have rendered M8 refractory to phosphatase activity, we hypothesized that multi-site phosphorylation of the P-domain (Fig. 1B) controls M8 repression of Ato.

To analyze multi-site phosphorylation of M8, we adopted site-specific (ϕ C31) integration to enable controlled comparisons of Ala/Asp variants at multiple kinase sites. We chose the ϕ C31 site at 68E, one of 'moderate' expressivity. This site was chosen, because random insertions of M8-S159D elicit a near complete loss of the eye (Fig. 1B), which we reasoned is likely to be a limit phenotype and thus unlikely to reveal the contributions of phosphorylation of sites in addition to CK2. Accordingly, *scaGal4* expression of M8-S159D (from ϕ C31-68E) elicits a reduced eye of moderate severity (Fig. 1C, E). No eye defect is seen upon expression of M8-S159D with *h^{H10}Gal4*, or in relevant controls (Fig. 1D, E). Thus the M8-S159D insertion at ϕ C31-68E qualitatively mimics random insertions, described previously.

E(spl)-M8 harbors a highly conserved consensus site for MAPK

The P-domain of M8 contains four Ser residues, which are invariant in 12 *Drosophila* species and the stalk-eyed fly *Teleopsis* (Fig. 2A) that diverged ~100 MYA. The flanking Ser residues meet the strict consensus for CK2 and MAPK, and the intervening six residues contain two additional phosphoacceptors. Importantly, the CK2 and MAPK sites are conserved in mammalian HES6, but with three distinctions. First, an insertion of six additional residues widens the spacing of the CK2 and MAPK sites. Second, this insertion includes tandem Asp residues (DD motif, Fig. 2A), which can often bypass the need for phosphorylation. Third, mouse HES6 replaces a Ser with Pro in the linker, suggesting that this site may not be vital to regulation of human HES6.

The full analysis of the contributions of all four Ser residues of M8 with Ala and/or Asp would necessitate ≥ 64 variants, beyond the scope of a single manuscript. We focused on the MAPK site, as this protein kinase mediates the effects of EGFR signaling, a pathway implicated in the birth of patterned R8s, and because murine HES6 is modified by CK2 and MAPK (Belanger-Jasmin et al., 2007; Gratton et al., 2003b). The developmental roles of HES6 phosphorylation remain unknown. Given the divergence time between Dipterans and mammals (Fig. 2A), we hypothesized that MAPK is important in controlling M8 activity.

We previously reported that a variant refractory for CK2 (M8-S159A) does not mimic wild type (unmodified) M8, but elicits a rough eye due to dominant-negative (DN) activity (Karandikar et al., 2004). We thus refrained from mutating the MAPK site on a backbone with an Ala at the CK2 site. A backbone of wild type M8 or M8-S159D (Fig. 2B) was used to generate three Ala/Asp variants of Ser151, the MAPK site. M8-S151A+S159D is refractory to MAPK but mimics CK2, and should reveal if these two kinases cooperate to control M8 activity or act redundantly. M8-S151D is a MAPK-mimic that should render M8 activity independent of EGFR/MAPK signaling. The third, M8-S151D+S159D, is a dual kinase

mimic that should render M8 activity independent of CK2 and MAPK. In a yeast two-hybrid assay, all variants interact robustly with Groucho (Gro, Fig. 2B), which is essential for M8 repressor activity in vivo.

An intact MAPK site is essential for activity of the CK2 mimic M8-S159D

We first compared the activity of M8-S159D (CK2 mimic) and M8-S151A+S159D (refractory to MAPK but mimics CK2). Unlike M8-S159D, no reduced eye was seen upon expression of M8-S151A+S159D with *scaGal4* (compare Fig. 3A to Fig. 1C). Two findings argue against a defective construct or instability of M8-S151A+S159D protein in vivo. This variant inhibits development of the IOBs (data not shown) and strongly elicits the loss of macrochaetes (MC's) and microchaetes (mc's) with potency similar to the CK2 mimic M8-S159D (Fig. 3B, C). These effects on the MC's/mc's reflect expression of *scaGal4* in proneural clusters that give rise to these bristle types. Thus an intact MAPK site is essential for M8 repressor activity in the eye, but not the bristle.

We next tested the MAPK-mimic (M8-S151D) and the dual-kinase mimic (M8-S151D+S159D). However, both variants elicited embryonic lethality when expressed with *scaGal4*. Lethality was seen even in crosses conducted at 18C where Gal4 activity is often attenuated (Fig. 3D). To circumvent this problem, we used *109-68Gal4*, a weaker stage-2/3 driver. Flies expressing all four M8 variants were viable and elicited a range of eye defects. Expression of the CK2 mimic (M8-S159D) elicited a rough eye and loss of the IOBs (Fig. 4A), but did not significantly reduce eye size (compare to Fig. 1C). This lack of a reduced eye reflects moderate expressivity from the ϕ C31-68E site (Fig. 1B, C) combined with the weaker driver *109-68Gal4*. The rough eye of M8-S159D was abrogated by the introduction of an Ala at the MAPK site (Fig. 4D), although loss of IOBs remained (data not shown). In contrast, the MAPK-mimic (M8-S151D) or the dual-kinase mimic (M8-S151D+S159D) elicited a strong loss of the eye with almost equal severity (Fig. 4B, C) demonstrating the

importance of the MAPK site in controlling M8 activity. No eye/IOB defects are seen in *109-68Gal4/+* flies or the four M8-transgenic lines on their own (inset in Fig. 4E). As *109-68Gal4* is active in PNCs that give rise to bristles, we also quantified loss of the MCs. Although attenuated in severity when compared to results with *scaGal4* (Fig. 3B, C), expression of all four variants with *109-68Gal4* elicited MC loss with similar potency (Fig. 4A', B', C', D'). The lack of effects on the mcs reflects weaker strength of *109-68Gal4*. Together, these studies reveal that the MAPK site is important for M8 activity in the eye, but not the bristle.

M8 variants with a modified MAPK site perturb R8 specification

We next determined if M8 variants perturb birth of R8s and secondary photoreceptors by staining for Senseless (Sens) and ELAV, respectively. The reduced eye upon expression of the CK2-mimic M8-S159D with *scaGal4* (Fig. 1C) reflects strong loss of R8s (Sens+ cells), and many of the R8s that emerge from the MF poorly sustain Sens expression, and thus inefficiently recruit secondary (ELAV+) photoreceptors (for example see Fig. 7A').

As the expression of MAPK variants with *scaGal4* was lethal (Fig. 3D), we used *109-68Gal4* to enable analysis of all M8 variants. Consistent with the wild type eye, *109-68Gal4/+* flies display proper Sens expression/maintenance and ELAV staining akin to wild type discs (data not shown). Expression of the CK2 mimic M8-S159D elicited the weakest effects (Fig. 5A) wherein a few R8s failed to maintain Sens expression and poorly recruited ELAV+ cells. Consequently, a few regions of the eye disc are devoid of closely juxtaposed ELAV clusters (circled region in Fig. 5A'), as compared to control discs. These R8s are likely removed by apoptosis, a default fate for uncommitted cells, contributing to the 'rough eye' of M8-S159D expressing flies (Fig. 4A). No such defects are seen with M8-S151A+S159D (Fig. 5B,B'), confirming that an Ala residue at the MAPK site renders M8 inactive in the eye even in the presence of an activating mutation at the CK2 site. In

contrast, discs expressing the MAPK mimic M8-S151D or the dual-kinase mimic M8-S151D+S159D display the inconsistent maintenance of Sens levels along the AP axis in a greater proportion of R8s (Fig 5C, D). Many of these R8s also poorly recruit secondary photoreceptors, such that numerous clusters contain less than (the normal) 1Sens+7ELAV cells, characteristic of discs from *109-68Gal4/+* or wild type flies (Fig. 5C',D'). Moreover, significant regions of the eye disc altogether lack Sens+Elav clusters, a result consistent with the reduced eye. Thus the reduced eyes of the variants described in Fig. 4 reflect defective birth and survival of R8s, which consequently impairs secondary photoreceptor recruitment.

An Asp residue at the MAPK site elicits inappropriate earlier activity of M8

We next tested if an Asp at the MAPK site renders M8 prematurely active, i.e., at stage-1 of the MF. This test was conducted because M8* (lacking the 'cis'-inhibitory CtD) elicits a strong loss of the eye at stage-1, whereas the CK2 mimic M8-S159D is without effect (Fig. 1B, D). As (endogenous) MAPK is normally activated at stage-2/3 of the MF where R8s are selected by lateral inhibition, we reasoned that an Asp at the MAPK site might well bypass the need for EGFR/MAPK signaling, and exhibit premature activity at stage-1, in a manner akin to M8*.

Indeed, the MAPK-mimic (M8-S151D) and the dual-kinase mimic (M8-S151D+S159D) both elicit a reduced eye upon expression at stage-1 (with *h^{H10}Gal4*) with similar potency (Fig 6A, B), and perturb the ommatidial lattice and the position of the IOBs (Fig. 6A', B'). No reduced eye was observed with M8-S151A+S159D (mimics CK2, but not MAPK), and neither did this variant elicit defects in the ommatidial lattice or the position of the IOBs (Fig. 6C, C'). These data seem consistent with the possibility that both CK2 and MAPK are needed for proper activation of M8 in a timely manner at stage-2/3; this regulatory system is bypassed by Asp mutations or by deletion of the CtD.

Altered EGFR signaling modulates M8 variants with an intact MAPK site

Given the importance of the MAPK site for M8 activation, we directly tested for a role for EGFR signaling. We used *egfr^{f24}*, a loss of function allele, which does not perturb the eye/R8s in the heterozygous state (data not shown) but is lethal when homozygous. We reasoned that if EGFR/MAPK signaling activates M8, then halved EGFR levels should attenuate the amount of active MAPK, and result in diminished phosphorylation of M8. If so, M8-S159D (mimics CK2 but an intact MAPK site) should become hypo-phosphorylated in *egfr^{f24}/+* flies, thereby dampening the severity of its reduced eye. In contrast, the MAPK-mimic (M8-S151D) and the dual-kinase mimic (M8-S151D+S159D) should be refractory to halved EGFR signaling by virtue of the Asp residue at the MAPK site.

Both of the suggested predictions bear out. The CK2 mimic M8-S159D was expressed with *scaGal4*, given the significant effect on the eye with this stage-2/3 driver. Compared to its effects in an *egfr⁺* background (Fig. 7A), the severity of the reduced eye of M8-S159D is significantly attenuated in *egfr^{f24}/+* flies (Fig. 7B), a result supported by quantitative analysis of eye size (Fig. 7C). Staining of eye discs reveals the cell specificity underlying the restoration of eye size in the *egfr^{f24}/+* background. Expression of the CK2 mimic M8-S159D in *egfr⁺* flies (Fig. 7A') elicits strong loss of patterned R8s (Sens+ cells), impairs R8 survival (maintenance of Sens levels) and results in diminished recruitment of secondary photoreceptors (ELAV+ cells). These R8 defects are strongly suppressed in an *egfr^{f24}/+* background (Fig. 7B' and B'').

A similar analysis was conducted with the MAPK-mimic (M8-S151D) and the dual kinase mimic (M8-S151D+S159D), but utilized the driver *109-68Gal4* due to lethality upon expression with *scaGal4* (see Fig. 3D). With both of these variants the severity of the reduced eye was indistinguishable in *egfr⁺* versus *egfr^{f24}/+* backgrounds, qualitatively (compare Fig. 7D, E to Fig. 4B, C) as well as quantitatively (Fig. 7F, graph includes values

from Fig. 3E). Staining of eye discs revealed that these two variants elicit R8 defects indistinguishable from those described earlier in *egfr⁺* flies (Fig. 5, and data not shown). Thus eye/R8 defects of M8 variants with a phosphomimetic Asp at the MAPK site are insensitive to halved EGFR/MAPK signaling.

Deletion of the CK2 or MAPK site mimics Asp-variants of M8 at these two kinase sites

We next sought to test the contributions of the CK2 and MAPK sites to ‘cis-inhibition’. We were intrigued by the findings that the MAPK-mimic M8-S151D or dual-kinase mimic M8-S151D+S159D both elicited a reduced eye and R8 defects at stage-1 of the MF (Fig. 6) in a manner akin to non-inhibited and constitutively active M8* (see Fig. 1B). These studies did not, however, resolve if the MAPK site in conjunction with CK2 imposes cis-inhibition on M8, or if the similar stage specificity of the MAPK-mimic and M8* is purely incidental. Specifically, we sought to determine if modification by CK2+MAPK serves primarily as a conformational switch, rather than for binding of repressive cofactors (other than Gro) to the phosphorylated CtD of M8. For this, we asked if deletion of the CK2 or MAPK site renders M8 active, thereby mimicking the outcomes of Asp mutations at these kinase sites. We deleted just the consensus sites for these two kinases. The first, M8-ΔCK2 lacks the CK2 site S¹⁵⁹DCD, whereas the second M8-ΔMAPK lacks the MAPK site PLS¹⁵¹P (Fig. 8A).

Based on the ‘cis’-inhibition model and studies with Asp mutations at the CK2 and/or MAPK sites (Figs. 1, 3-7), we made three predictions. First, M8-ΔCK2 should resemble the CK2-mimic M8-S159D and elicit a reduced eye at stage-2/3, but not stage-1. Second, M8-ΔMAPK should mimic M8-S151D and elicit a reduced eye at stage-1 and stage-2/3. Third, the reduced eye of M8-ΔCK2 should be mitigated by halved *egfr* dosage, whereas that of M8-ΔMAPK should be insensitive.

Indeed, all three predictions bear out (Fig. 8). At stage-1, M8-ΔCK2 is largely

inactive, whereas M8-ΔMAPK elicits a ~50% reduction of the eye (Fig. 8B, C, D). In contrast, M8-ΔCK2 elicits a strong loss of the eye at stage-2/3 (Fig. 8E), whose severity is significantly attenuated in an *egfr^{f24}/+* background (Fig. 8F). This result is supported by quantitative analysis of eye size (Fig. 8G). As predicted, expression of M8-ΔMAPK at stage-2/3 elicits a reduced eye of comparable size in *egfr⁺* or *egfr^{f24}/+* backgrounds (Fig. 8H, I), results also supported by quantitative analysis of eye size (Fig. 8J). Thus deletion of the CK2 and MAPK sites elicits M8 activity and eye defects that closely mimic the effects of Asp mutations at these respective kinase sites. We conclude that the two flanking kinase sites participate in 'cis'-inhibition of M8, and that this control is bypassed by deleting kinase consensus sites within the P-domain (Fig. 8), the introduction of phospho-mimetic Asp residues or by the complete loss of the CtD, as with M8* (Fig. 1B).

PP2A may target the MAPK site during M8 regulation

Recent studies from our lab reveal a role for the phosphatase PP2A in birth of patterned R8s (Bose et al., 2014). Relevant to the studies described here, overexpression of *widerborst* (*wdb*), a PP2A regulatory subunit, rescues the eye /R8 defects of the CK2 mimic M8-S159D (Fig. 9A). No rescue was evidenced upon co-expression of *UAS-LacZ*, excluding the possibility that rescue reflects competition between two *UAS*-constructs for a limiting amount of Gal4 protein. The modulation of the eye defects of M8-S159D by *wdb* raised the prospect that PP2A targets the MAPK site, rather than that for CK2.

We thus tested if PP2A opposes the effects of EGFR/MAPK signaling, by asking whether co-expression of *UAS-Wdb* modulates the reduced eye of the MAPK-mimic (M8-S151D) and the dual-kinase mimic (M8-S151D+S159D). The reduced eye of either variant upon co-expression of *UAS-Wdb* (Fig. 9B, C) appeared similar to that in its absence (Fig. compare with Fig. 4B, C), a result supported by quantitative analysis of eye size (Fig. 9D). The eye sizes in the presence of ectopic Wdb were statistically similar to those in its

absence (note that Fig. 9D includes data values from Fig. 4). Hence, unlike the CK2-mimic M8-S159D, an Asp residue at the MAPK site renders M8 insensitive to Wdb, suggesting that this phosphatase might oppose M8 activation by EGFR/MAPK signaling. In summary, we conclude that the highly conserved MAPK site is important for activation of M8 in a spatially precise manner in the MF, and that this activation is antagonized by PP2A.

Discussion

The studies described here reveal that the highly conserved MAPK site in M8 is important for repressor activity in the developing eye, but appears to be dispensable during bristle development. Specifically, our studies have utilized Ala/Asp variants of the MAPK site in wild type (Figs. 1, 3, 4, 5, 6) and *egfr* mutant backgrounds (Fig. 7). These findings support an important role for EGFR/MAPK signaling in controlling M8 repression of Ato. In addition, our studies on deletion variants of M8 (Fig. 8) reveal that both kinase consensus sites contribute to 'cis'-inhibition, which is regulated by multi-site phosphorylation. Finally, our analysis of PP2A-Wdb (Fig. 9) raises the prospect that this phosphatase targets the MAPK site of M8. The conservation of sites for these two kinases in M7, M5 and My raises the prospect that this regulatory mechanism has a broader impact that has been realized. Together, our studies reveal that instead of acting merely in a dosage-dependent manner, distinct tissue-specific regulatory mechanisms control repression by E(spl) proteins.

Multi-site phosphorylation exerts spatial control over M8 activation in the eye

The studies we describe here expand on our understanding of the role of the conserved P-domain in M8. Our data suggest a revised model wherein conversion of the 'cis'-inhibited state of M8 to an active repressor of Ato depends upon phosphorylation of the CK2 and MAPK sites, and that the precise (restricted) activation of this key Ato repressor at stage-2/3 of the MF occurs in response to EGFR signaling (Fig. 10). If so, it would suggest that EGFR signaling, which is required to activate MAPK at stage-2/3, is crucial for inhibitory

effects of Notch to occur properly, and without which R8 patterning and eye development would be perturbed. The emerging role for PP2A in regulation of M8 activity raises the prospect that this phosphatase may play two potential roles, not necessarily mutually exclusive. By antagonizing modification at the MAPK site, PP2A may control the amount of active (non 'cis'-inhibited) M8, or it may act to control M8 inactivation immediately upon the selection of single R8s. The former regulation may thus impose control over '*signal amplitude*' (amount of active M8), whereas the latter would control '*signal duration*'. Our studies, which do not discriminate between these possibilities, nevertheless reveal that repression by E(spl)-M8 is controlled by PTM.

We note that our analyses do not reveal the epistatic relationship between CK2 and MAPK, because demonstration of the order of M8 phosphorylation necessitates isoform- and phospho-specific antibodies. Our efforts to raise antibodies that recognize specific phosphorylated forms of M8s CtD have not been successful, as they display cross-reactivity not only with unmodified M8 but with other members that share sequence similarity in their CtDs (M5, M7, My). In light of the close conservation of the CK2 and MAPK sites in the E(spl) proteins M7, M5 and My, the likelihood is high that this manner of regulation may have a broader impact in tissues where these bHLH proteins function. We discuss the implications of our findings in the context of two signaling pathways, Notch and EGFR, which are vital not only to R8 ontogeny, but also to subsequent stages of retinal histogenesis.

Notch and the specification of the R8 cells

The key role of Notch as *the* driver for lateral inhibition is indisputable. Detailed and elegant studies have shown that loss of Su(H) or the E(spl)C leads to the specification of excess ('twinned') R8s, a hallmark of impaired lateral inhibition. The developmental outcome of loss of Notch is more complicated, in particular at the onset of retinal

histogenesis because this receptor plays dual roles during birth of the R8 cells. Notch is first needed for 'proneural enhancement', a positive feedback loop that is necessary to upregulate Ato levels in an Ato+Da-dependent manner, after which it is needed for lateral inhibition. The mechanism underlying these two antagonistic functions has, to our knowledge, remained undefined at the molecular level. Our studies suggest that spatially controlled phosphorylation of M8 may introduce a '*time-delay-circuit*'. Without this delay Ato upregulation would be impaired, and Ato levels in the pre-R8 clusters would not achieve the threshold necessary to adopt the R8 fate. The strong loss of Ato upregulation that is seen with E(spl)D or upon stage-1 expression of M8* would be consistent with our model, and underscore the prescient suggestion of Giebel and Campos-Ortega that this region (the CtD) may impose control over repression.

EGFR and the specification of the R8 cells

While there are numerous lines of evidence for the contribution of MAPK in retinal determination, the role for the EGFR pathway in R8 selection is still unclear. The EGFR/MAPK signaling pathway has been suspected to play a role in R8 patterning. For example, *egfr*^{-/-} clones display the specification of 'twinned' R8s, a phenotype also manifest in clones of the downstream effectors Ras and Raf (Yang and Baker, 2001). A similar analysis of MAPK^{-/-} clones has been stymied due to cell lethality. Nevertheless, we note with interest that a 'twinned' R8 phenotype is not seen in clones of *pointed* (*pnt*), the nuclear effector of EGFR signaling, or *anterior open* (*aop* or *yan*), that acts as a repressor (Yang and Baker, 2003). Moreover, active dual-phosphorylated MAPK (dp-ERK) is detectable in cells at stage-2/3 of the MF (Kumar et al., 1998), and this staining is lost in *egfr*^{-/-} clones (Lesokhin et al., 1999), suggesting that MAPK activation at this stage of the MF is not being driven by another RTK. Surprisingly, and unlike the canonical mode of EGFR/MAPK signaling, dp-ERK does not translocate to the nucleus and instead appears cytoplasmic,

referred to as the 'cytoplasmic-hold' (Kumar et al., 2003). This is intriguing, because in this location active-MAPK would not be able to phosphorylate either Pnt or Yan, which are constitutively nuclear proteins (Shilo, 2003). The identity of the proteins targeted by this active cytosolic MAPK has remained unknown. Do these studies suggest that active MAPK may act to regulate M8 in the cytosol and that this activated protein then enters the nucleus to mediate repression? While the answers to this question are presently unknown, we note that the default location of the M8 homolog HES6 is cytosolic in the myogenic program (Gao et al., 2001), and nuclear localization of this repressor is closely coupled to induction of differentiation, suggesting that the nucleus is not the default location of (at least) this HES repressor.

Then how does MAPK phosphorylation precisely affect M8 activity? We have previously proposed that M8 is an autoinhibited protein, an intramolecular restraint upon repressor capabilities that keeps the protein in an inactive state (Kahali et al., 2010b; Karandikar et al., 2004). Surprisingly, the deletion of the CK2 site, which in a sense mimics the phosphorylation, leads one to propose therefore the simplest interpretation that it is the CK2 site that itself imposes some protein-protein contact that is responsible for controlling the onset of repression. However, this variant at an earlier stage of the furrow fails to perturb the eye field, which now necessitates a simultaneous deletion of the MAPK site indicating that neither the CK2 nor the MAPK phosphorylation is likely to act as a scaffold or serve as a basis for secondary modulation. In fact, these phosphorylations may be required purely at a level of intramolecular protein contact. If so, it is reasonable to suggest that both CK2 and MAPK act to restrain autorepression, and the data presented with Asp replacement at both kinase sites as well as the deletion of either kinase site, would suggest that the protein is under dual control. We think that dual control is a mechanism to allow for the precise regulation of the protein. One question that has always remained unanswered is

what triggers the modification of M8 by CK2? This kinase does not respond to extracellular or intracellular signaling pathways, but clearly does not appear to modify M8 on its own because co-expression of CK2 and M8 does not pheno-copy the effects of the Asp. Our studies do not allow us to tell whether CK2 modification acts prior to that of MAPK. In any event however, given that MAPK activation is exclusively dependent on EGFR pathway activation, it therefore is reasonable to suggest that the gate-keeping function could well be mediated by EGFR.

These studies for the first time demonstrate a developmental context under which the MAPK site in M8 protein is of biological consequence. As shown in the last figure (Fig 10), co-ordinated functions of several kinases (CK2 and MAPK) and phosphatase (PP2A) control the regulatory output of M8 repression. These PTMs may be a way to balance the amplitude of the M8 signal or the duration. While phosphorylation by CK2 and MAPK allows for precise on-set of repressor activity of M8 and thus for R8 selection, de-phosphorylation by PP2A ensures a rapid inactivation of the protein.

Materials and Methods

Construction of M8 variants: M8 variants with Ala/Asp substitutions at the known phosphorylation site for CK2 (Ser159) and/or that predicted for MAPK (Ser151) were generated by PCR based site-directed mutagenesis. M8 variants harboring deletions of the CK2 (**S159DCD**) or MAPK (**P149LSP**) consensus motifs were generated by inverse-PCR. In both cases (Ala/Asp or deletion variants), the forward and the reverse primers incorporated EcoRI and BamHI sites 5' and 3' to the coding region, respectively. The forward primer incorporated a KOZAK consensus (CAAC) immediately 5' to the ATG codon for efficient expression. All constructs were fully sequenced to confirm the presence of only the intended mutations.

Germ line transformation: The Ala/Asp variants of M8 targeting the CK2 and/or MAPK sites were cloned into the vector pUAST-attB, whereas the deletion variants were cloned into pUAST (Brand and Perrimon, 1993). Transgenic lines were generated using a commercial embryo injection facility (BestGene, Inc.). All pUAST-attB constructs were integrated using the ϕ C31-docking site at 68E on the third chromosome (Bateman et al., 2006). The transgenic lines were verified by PCR amplification and sequencing of the PUAST-attB construct, and a single molecularly defined line was used in the studies. For the pUAST-constructs, w⁺ progeny were identified and the insertion site was mapped by standard approaches using chromosomes harboring dominant markers. In the case of pUAST constructs, 10 independent insertions of M8- Δ CK2 and M8- Δ MAPK were generated, and of these ≥ 5 have been used in the studies.

Biochemical analysis: Protein-protein interactions were analyzed by the LexA-based version of the yeast interaction trap. The bait and prey constructs were expressed as C-terminal fusions with DNA-binding domain of LexA and the activation domain (AD) of B42,

respectively. The yeast strain EGY048p was used to evaluate protein interactions, as described (Trott et al., 2001).

Fly stocks: The Gal4 drivers were obtained from Bloomington Stock Center (denoted by the prefix B). These drivers are *109.68Gal4* (B6479), *h^{H10} Gal4* (Fischer-Vize et al., 1992) *scaGal4* (Giebel and Campos-Ortega, 1997). The DER mutant line *egfr^{f24}* (B6500) was also obtained from Bloomington Stock Center. *wdb^{EP3559}* flies were a gift from Amita Sehgal (U. Penn).

Eye and bristle phenotypes: All crosses were performed on standard Yeast-Glucose medium at 24°C, unless indicated otherwise. Fly heads were dehydrated by sequential passage through a graded alcohol series for 24 hours each (25%-50%-75%-100%), and finally passed through Hexamethyldisalinaze. Heads were mounted on EM stubs, dried for 24 hours, sputter coated with gold and examined with a JEOL-6400 scanning electron microscope at an accelerating voltage of 10-20kV. Images were processed with Adobe Photoshop and collated in Adobe Illustrator. The reduced eye was quantified from ~20 adult flies that were photographed using a Leica MZ16 stereomicroscope equipped with a Leica DFC-480 digital camera. Facet numbers were counted from TIFF images. A similar approach was used to determine bristle phenotypes. Eye size (facet counts) and bristle defects were statistically analyzed using Student's T-test.

Immunostaining and confocal microscopy: Eye imaginal discs were isolated from late third instar larvae and processed as described (Kavler et al., 1999) with minor modifications. After isolation, discs were fixed in 4% paraformaldehyde in 1x phosphate buffered saline (PBS) for 45 minutes at 4°C. After fixation, discs were washed three times with PBS containing 0.3% Triton X-100 (PBS-TX) for 15 minutes each. The discs were incubated for 12-14 hours at 4°C in PBS-TX containing primary antibody. The following

antibodies were used; guinea pig anti-Sens (gift from Hugo Bellen, HHMI-Baylor) at a dilution of 1:2000 and mouse anti-ELAV (DSHB, Iowa) at a dilution of 1:1000. Following primary antibody binding, eye discs were washed three times with PBS containing 0.3% Triton X-100 and then incubated with secondary antibody at room temperature for 2-3 hours. The secondary antibodies (Molecular Probes) used for are, goat anti-guinea pig-IgG coupled to Alexa Fluor 633 (1:1000) and goat anti-mouse-IgG coupled to Alexa Fluor 488 (1:1000). The discs were mounted in 60% glycerol, and viewed on an Olympus FluoView (FV100) for confocal imaging. Images were acquired every 1µm along the apicobasal axis of the discs and then compressed as a Z-stack without the removal of any layers. Compressed Z-stacks were exported as TIFF files and collated in Adobe Illustrator.

Fig. 10: MF specificity of M8-S159D.

(A) Schematic representation of the morphogenetic furrow (MF) depicting the expression domains of different Gal4 drivers. The cell fates at different stages, spanning the MF are represented in colored shades. (B) The eye phenotype of CK2 variants (random insertion lines) driven by two different drivers, *scaGal4* and *109-68Gal4* at stage-2/3 of the MF. (C,D) Scanning EM of adult eye at a magnification of 200x. (C) Overexpression of CK2 phosphomimetic form M8-S159D, at stage-2/3 of the MF perturbs the eye. (D) M8-S159D fails to generate any eye defect at stage-1 of the MF. (E) Ommatidial (facet) counts were determined in ≥25 of the indicated genotypes. Asterisk denotes P-value < 0.001.

Fig. 11: Conservation of Ser residues in the P-domain of E(spl)-M8.

(A) The functional domains of E(spl)-M8 are represented by a basic domain (DNA binding), an HLH domain (dimerization), a second HLH region, the Orange domain (interaction with Ato/ASC) and a C-terminal WRPW motif (Gro-binding). While these domains are highly conserved among all the E(spl) members, the length/sequence heterogeneity is largely confined to the C-terminal domain (CtD), the region between Orange and WRPW. The CtD has a phosphorylation domain (P-domain) with several conserved Ser residues. The mammalian Hes6 has additional Asp residues in the CtD (black arrow) (B) Sequence alignment of P-domain of E(spl)-M8 homologs in 12 *Drosophila* species, Stalk-eyed fly (*T. dalmani*) and the mammalian homolog of E(spl)-M8 in mouse Hes6 and human Hes6. The blue and yellow shaded boxes meet the consensus for MAPK and CK2 phosphorylation motifs respectively. Conserved phosphorylatable Ser residues are represented in bold red. Phylogenetic relationship of E(spl)-M8 of all the 12 *Drosophila* species are represented at

the right side of Panel B. (C) The Φ c31 M8 phospho variants used for the study. M8-S159D represents the CK2 mimic and M8-S151D represents the MAPK mimic. M8-S151D+S159D mimics CK2+MAPK dual phosphorylated form. M8-S151A+S159D is the CK2 phosphomimetic and MAPK refractory form of the protein. Two-hybrid interaction of the M8 variants with Gro (co-repressor) were measured as Lac Z activity (Miller Units) and represented as “+” in the table.

Fig. 12: MAPK refractory but CK2 mimic does not perturb the eye.

(A) M8-S151A+S159D (CK2+no MAPK) fails to generate any eye defect at stage-2 of the MF. (B, C) Loss of MC's on the heminotum displayed by M8-S151A+S159D and CK2 mimic M8-S159D. (D) MAPK mimic (M8-S151D) and dual-kinase mimic (M8-S151D+S159D) elicit embryonic lethality (EL) when driven at stage-2/3 of MF by *scaGal4*.

Fig. 13: Secondary phosphorylation enhances M8 activity.

(A-D) Adult eye phenotype of all the phospho-mimetic variants driven by *109-68Gal4* at stage-2/3 of the MF. (E) Ommatidial (facet) counts were determined in ≥ 20 of the indicated genotypes. Asterisk denote P-value < 0.001 . (A'-D') The phosphor-variants elicited MC loss. (F) Heminotum MC loss was determined in all the four variants. Asterisks denote P-value < 0.001 .

Fig. 14: Phosphomimetics perturb R8 birth and differentiation.

Eye disc of the aforementioned genotypes were immunostained with α -Sens (magenta) and α -ELAV (green) to determine R8 differentiation and secondary photoreceptor recruitment respectively. Arrow indicates direction of MF progression. (A) CK2-mimic M8-S159 shows absence of closely juxtaposed ELAV+ cluster. Note that MAPK mimic M8-S151D (C) and CK2+MAPK dual mimic M8-S151D+S159D (D) random patches of non-specified and non-differentiated retinal tissue. On the contrary, M8-S151A+S159D (B) shows uniformity in Sens+ELAV clusters, akin to WT disc (data not shown).

Fig. 15: MAPK mutation causes precocious M8 repressor activity at stage-1 of MF.

(A-C) Adult eye phenotypes of different M8 variants at stage-1 of the MF. Both M8-S151D (A) and M8-S151D+S159D (B) perturb the eye field whereas M8-S151A+S159D (C) fails to generate any eye defect at stage-1 of the MF. (A'-C') 1000x magnification of the corresponding genotypes in panels (A-C). There are extra IOBs specified for MAPK mimic (A') as well as the MAPK+CK2 mimic (B'). The CK2+no MAPK mimic (C') fails to specify extra IOBs. Ommatidial (facet) counts were determined in ≥ 20 adult flies. Asterisk denote P-value < 0.001 .

Fig. 16: Decreased EGFR levels rescue reduced eye of CK2 mimic only.

(A-B) Scanning EM of adult eye at a magnification of 200x. (A) Overexpression of CK2 phosphomimetic M8-S159D form at stage-2/3 of the MF at an *egfr^{+/+}* background perturbs the eye. (B) M8-S159D when expressed at *egfrf24/+* background restores the eye field. Graph shows ommatidial count of the adult eyes. Data labeled A and B correspond to the

adult eyes shown in panel (A) and (B). Facet count was determined in ≥ 20 flies. Asterisk denotes P-values < 0.001 . (A'-B') Eye discs of genotypes indicated in A and B were immunostained with α -Sens (magenta) and α -ELAV (green) to assess R8 differentiation and secondary photoreceptor recruitment. Panel (B'') shows a magnified image of panel (yellow box in B') to highlight the rescue of differentiated R8s and Elav clusters. (D,E) Overexpression of MAPK phosphomimetic M8-S151D and CK2+MAPK dual mimic M8-S151+159D at stage-2/3 of the MF at *egfr^{f24}/+* background fails to restore the eye field. Facet count was determined in ≥ 20 flies. Data labeled D and E correspond to the adult eyes shown in panel (D) and (E). Asterisk denotes P-values < 0.001 .

Fig. 17: Deletion variants of M8 perturb eye development.

(A) Schematic representation of the P-domain deletion variants of M8. M8- Δ CK2 lacks the CK2 motif and M8- Δ MAPK lacks the MAPK motif. The deletion variants were expressed at stage-1 of the MF driven by *h^{H10}Gal4*. (B-C) M8- Δ CK2 fails to generate any overt eye defect (B) whereas M8- Δ MAPK generates a reduced and rough eye phenotype (C). (D-E) At an *egfr*^{+/+} background, M8- Δ CK2 overexpression driven by *scaGal4* leads to a rough and reduced eye (D). The eye defect of M8- Δ CK2 is rescued at a halved dosage of *egfr*^{f24/+} background (E). Similar studies involving M8- Δ MAPK was precluded as *scaGal4* driving the deletion variant led to embryonic lethality (see inset Fig 8). (F-G) In order to circumvent the problem, weaker driver *109-68Gal4* was used which resulted in a rough and reduced eye phenotype (F) at an *egfr*^{+/+} background. The eye defect of M8- Δ MAPK at stage-2/3 was not rescued in a halved dosage of *egfr*^{f24/+} background (G). The graphs at the bottom panel are for the corresponding genotypes. Ommatidial (facet) count was determined in ≥ 20 flies. Asterisk denotes P-values < 0.001.

Fig. 18: MAPK site in M8 may be a target for modulation by PP2A.

(A) Overexpression of *widerborst* (*wdb*) rescues the eye defects of CK2 mimic M8-S159D (adapted from (Bose et al., 2014)). Scanning EM of the adult eye at 200x magnification (B-C). Co-expression of Wdb fails to rescue the reduced eye phenotype due to overexpression of (B) M8-S151D and (C) M8-S151D+S159D. Graph shows ommatidial (facet) counts of the adult eyes. Data labeled B and C correspond to the adult eye shown in panels B and C. The +/- controls for each corresponding genotype are adapted from Fig 3. Facet counts were determined in ≥ 20 flies, and asterisk denotes P-values < 0.001 .

Fig. 19: Phosphorylation and dephosphorylation regulates M8 repressor activity during retinal neurogenesis.

Model for M8 regulation. In the auto-inhibited state, the CtD loops back and blocks the HLH and/ Orange domain thereby prohibiting M8 interaction with Ato. CK2 and MAPK phosphorylation converts M8 into an active repressor for Ato, whereas PP2A mediates inactivation, either by a conformational change or by marking the protein for modifications by unknown targets for proteasomal degradation. The cartoon in the right represents the spatio- temporal regulation of M8 by phosphorylation and dephosphorylation by all these different factors in a 48-hr window of MF progression. As all the photoreceptors at stage-1 slowly starts accumulating Ato, M8 is produced at stage-2/3; undergoes post-translational modification (PTM) by CK2 and MAPK and restricts Ato in R8 photoreceptors. Immediately after, PP2A restricts M8 activity either by modulating the duration or amplitude of the repressor protein thereby preventing any abnormal repression.

CHAPTER 3: Identify the role of P-domain in autoinhibition of M8

Abstract

During autoinhibition, the C-terminal domain (CtD) of M8 mediates a '*cis*' interaction with the residues of the bHLH through the Orange domain. At the time of R8 specification, M8 undergoes phosphorylation by CK2 at the CtD, which is a necessary step for repressing the proneural protein Atonal. The CK2 site resides in a region that conserves additional Ser residues. This region of conserved Ser residues has putative sites for modifications by other kinases and has been termed as the P-domain (phosphorylation domain). Using deletion variants of specific kinase motifs and the entire P-domain, we demonstrate that this Ser-rich region is necessary for autoinhibiting M8. We have also tested this model for inhibition by co-expressing the CtD-peptide, which rescues the eye and the bristle defects of the deletion variants. This key regulation by the P-domain is bypassed by the E(spl)D mutation encoding the truncated protein M8*, which inhibits Ato precociously. Additionally, we provide evidence that the P-domain may undergo modulation by Slmb, a subunit of E3 ligase, which regulates proteasomal degradation. Together these studies provide evidence that the P-domain of CtD has a role in autoinhibition of M8 and also assists in protein clearance in a phosphorylation dependent manner.

Introduction

The Notch signaling pathway is highly conserved in invertebrates and vertebrates, and is involved in induction as well as restriction of cell fates. The functions of Notch have been exceptionally well analyzed during neurogenesis, where it mediates lateral inhibition to regulate dichotomy between neural versus epidermal cell fate (Artavanis-Tsakonas et al., 1999; Baonza and Freeman, 2001; Bray, 2006). The two sensory organs of *Drosophila*, which have been studied extensively to understand the mechanism of lateral inhibition include the compound eye and the mechanosensory bristles, such as the macrochaetes (MC's), microchaetes (mc's) and the interommatidial bristles (IOB's) (Modolell, 1997; Modolell and Campuzano, 1998; Simpson, 1990). Although different in structure, eye and bristle development involves similar genes and cell fate/lineage decisions. Studies involving these two model structures have led to our present day understanding of the core components of the Notch pathway and how it regulates cell fate specification.

At the onset of neurogenesis, proneural basic-helix-loop-helix (bHLH) activators encoded by *atonal* (*ato*) or the *achaete-scute complex* (*ASC*) is expressed in groups of cells, called proneural clusters (PNCs) (Heitzler et al., 1996; Jarman et al., 1995; Jarman et al., 1994; Kiefer, 2005; Skeath and Carroll, 1991). While *ato* is required for eye development, *ASC* expression is critical for bristle morphogenesis (Calleja et al., 2002; Campos-Ortega, 1998; Jarman et al., 1995; Jarman et al., 1994). The activities of these proneural proteins drive the formation of the PNCs, in which all cells have somewhat equal potential to adopt the neural fate (Dambly-Chaudiere and Vervoort, 1998; Frankfort and Mardon, 2002; Gibert and Simpson, 2003; Hsiung and Moses, 2002). From each PNC, however, only a single cell becomes the neural progenitor, the 'founding' R8 photoreceptor in case of the eye or the bristle sensory organ precursor (SOP), while the others are redirected to an alternative fate. This restriction of the R8/SOP from a PNC is termed lateral inhibition (Axelrod, 2010; Castro et al., 2005; Simpson, 1990). During this process, the presumptive R8/SOP activates Notch

in the adjacent cells of the PNCs (the non-SOPs/R8s). Specifically, activation of Notch results in the cleavage and nuclear translocation of its intracellular domain (N^{icd}), where it associates with and activates *Suppressor of Hairless* (*Su(H)*) leading to the transcription of a family of basic Helix-loop-Helix (bHLH) repressors, collectively called the *Enhancer of split Complex* (*E(spl)C*) ((Bailey and Posakony, 1995; Lecourtois and Schweisguth, 1995) (Bray, 1997; Delidakis and Artavanis-Tsakonas, 1991). The *E(spl)* repressors complex with the co-repressor Groucho and antagonize Ato/ASC to restrict the R8/SOP fate in all but one cell of a PNC.

Despite our understanding of the Notch pathway components and the mechanism of activation of *E(spl)* genes, our knowledge on the roles of functional domains of these repressors and their mode of regulation still remains unclear. Given their conserved structure, a long-standing view is that these proteins are functionally redundant, and that repression by these proteins is purely a reflection of their dosage. A body of genetic evidence contradicts this view. For example, while loss of *E(spl)C* elicits ectopic R8s/SOPs, overexpression of the various *E(spl)* members only affected bristle-SOP fate but R8 specification remained largely unperturbed (Giebel and Campos-Ortega, 1997; Ligoxygakis et al., 1998; Nakao and Campos-Ortega, 1996). Of the three *E(spl)* members, M8, M δ and M γ which are expressed in the morphogenetic furrow (MF) during eye development, only M δ overexpression elicit loss of R8's (Ligoxygakis et al., 1999; Nagel et al., 1999). The first line of evidence that protein dosage of bHLH repressors is insufficient for inhibition of R8 cell came in light when *E(spl)D*, a unique dominant allele of m8 elicited dominant loss of R8s and the eye (Kahali et al., 2009a; Nagel and Preiss, 1999).

The dominant eye defect elicited by *E(spl)D* allele can be attributed to its enhanced interaction with and antagonism of Ato (Nagel and Preiss, 1999). It was paradoxical that a similar result could not be recapitulated by overexpression of full length M8. Detailed study

revealed that the protein encoded by the *E(spl)D* allele is a truncated protein called M8*, which lacks 56 C-terminal residues (Nagel et al., 1999; Tietze et al., 1992). It was this discovery that led to presciently suggest that the C-terminal domain (CtD) of M8 might have regulatory influence on its repressor activity (Campos-Ortega, 1997). Later studies show that the CtD of M8 is phosphorylated by CK2 at a specific motif, a region missing in M8* (Trott et al., 2001). As a consequence of this post-translational modification (PTM), M8 undergoes a conformational change, which enables it to bind and antagonize Ato. Replacement of the CK2 phospho-acceptor (Ser¹⁵⁹) with a phosphomimetic Asp residue (M8-S¹⁵⁹D aka M8-SD) elicits exacerbated loss of R8s and the eye with a potency akin to *E(spl)D*. Moreover, M8-SD interacts with Ato with a binding strength equal to that of M8*. Consequently it was proposed that the CtD autoinhibits full length M8. Phosphorylation displaces this intra-molecular interaction permitting repression of Ato. This appears to be the case because co-expression of non-phosphorylated M8-CtD rescues the reduced eye of CK2-mimic M8-SD, even though the interaction would be in '*trans*'. A CtD with Asp at the CK2 site failed to rescue the R8s and eye defect (Kahali et al., 2009b). These results implicate CK2 as a regulator of R8 selection through the activation of M8 (Karandikar et al., 2004). Accordingly, loss of CK2 in otherwise wild type flies compromises lateral inhibition and elicits the specification of excess R8s from the IGs (Bose et al., 2006).

In case of M8, M5, M7, My and the human homolog of *E(spl)* protein, HES6, the CK2 site is located in a region termed as the P-domain, which reveals the presence of additional conserved Ser residues. This Ser rich region represents consensus sites for phosphorylation by MAPK, CK1 and GSK3. In case of HES6, the PxSP motif in its tail region has already been shown to be a target for MAPK, an effector of EGFR pathway, although its developmental consequences are presently unknown (Belanger-Jasmin et al., 2007). Although, mutations in CK1 or GSK3 have no known consequences in R8

patterning, the role of EGFR in R8 specification has long been suspected. The close clustering of CK2 and MAPK sites on the CtD raises questions on their role on regulating repressor activity of M8. Furthermore, recent studies from our lab have shown that dephosphorylation of M8 by phosphatase PP2A may play a role in antagonizing Notch signaling (Bose et al., 2014). It is speculated that PP2A may control the amount of active (non-inhibited) M8, or it may act to control M8 inactivation immediately upon the selection of single R8s, providing a rapid and potent mechanism to shut off M8. Additionally, a large-scale *Drosophila* protein-protein interaction screen has revealed supernumerary limbs (Slmbs) to be an interacting partner of M8 (Guruharsha et al., 2012). Slmb is the substrate specificity subunit of the SCF E3-ubiquitin ligase that targets proteins for degradation via the 26S proteasome. Modifications of Ser 154, 155 and 159 (CK1, GSK3 and CK2 sites respectively) in the CtD of M8 via phosphorylation may generate a Slmb-binding consensus motif, thereby eliciting formation of the 'Slmb-phosphodegron' which can be then targeted for degradation. These coordinated phosphorylation-dephosphorylation and the binding of Slmb for proteasomal degradation may be a method for regulating M8 activity during R8 specification.

In this chapter we have employed deletion variants of M8 protein to explain the autoinhibition model. We have used deletion variants of CK2-motif, MAPK-motif and P-domain motif to explain the role of multisite phosphorylation as a mechanism for controlling regulatory activity or degradation of M8. We have determined that the P-domain plays a role in regulating the activity of M8 during R8 specification. Our studies on the rescue of the eye and bristle defects of ectopic deletion variants with overexpression of the CtD-peptide are consistent with autoinhibition. Moreover, our studies also suggest that an intact P-domain is necessary for the formation of phosphodegron, which is a necessary step for degradation of M8. The implications of these findings are discussed (see discussion).

Results

Deletion variants and their differential roles at stage-1 of the MF

As stated in the introduction (see above), the CtD of M8 has been suggested to play a role in regulation of M8 during neurogenesis. Studies from the lab have shown that overexpression of the phosphorylatable CtD variant, M8-CtD, strongly represses the eye and bristle defects of ectopic M8-S159D at stage-2/3 of the MF. This shows that M8-CtD retains the autoinhibitory activity and is able to prevent the inappropriate repression of Ato by CK2 mimetic form M8-S159D. Similar autoinhibitory activity of phosphomimetic CtD variant M8-SD-CtD was not seen (Kahali et al., 2010b).

To further analyze the role of P-domain in autoinhibition, we have generated deletion variants of M8. We adopted random integration method to generate M8- Δ CK2, M8- Δ MAPK and M8- Δ PD deletion lines. We then characterized the in vitro interaction of the variants with co-repressor Groucho. In a yeast two-hybrid assay, all variants interact robustly with Groucho (Gro, Fig. 1B), which is essential for M8 repressor activity in vivo.

We next assessed if overexpression of the deletion variants elicit eye defect at stage-1 of the MF. The expressivity of the endogenous E(spl) genes do not correlate with stage-1. Hence, it was expected that overexpression of M8 deletion variants, which have bypassed the need for phosphorylation and is already primed for Ato inhibition, would show effect at stage-1 of the MF. On the contrary, M8- Δ CK2 overexpression at stage-1 (*h^{H10}Gal4*) is without effect (Fig. 1C). This stage-1 inactivity of M8- Δ CK2 was unexpected as Ato levels are lowest at this point of R8 birth. In contrast, M8- Δ MAPK indeed shows a reduced eye at stage-1 (Fig. 1D). This activity may reflect the necessity of phosphorylation of M8 at MAPK site in order to induce its repressor activity. M8- Δ MAPK bypasses the need for phosphorylation at the CK2 site and is ready to repress Ato at stage-1 of the MF. On the other hand, since M8- Δ CK2 still has an intact MAPK site, which cannot be phosphorylated at stage-1 of the MF due to lack of MAPK at that stage, fails to perturb the eye. It was

expected that M8- Δ PD would perturb the eye with more severity than M8- Δ MAPK, given it is lacking the entire P-domain, which may be attributed to the structural component of the autoinhibition model. Unfortunately, any assessment with M8- Δ PD at stage-1 was not possible owing to embryonic lethality of this deletion variant. Lethality was seen even in crosses conducted at 18C where Gal4 activity is attenuated (Fig. 1 inset)

All three-deletion variants elicit eye defect at stage-2/3 of the MF

We next compared the activity of the deletion variants at stage-2/3 of the MF. To eliminate position effects, multiple independent lines harboring deletion constructs were used. The expression of the UAS-constructs were driven with the enhancer trap *scaGal4*, that elicits Gal4 expression in stage-2/3 of the MF, where endogenous E(spl) mediates selection of R8 cells. The expression of M8- Δ CK2 with *scaGal4* elicits a reduced eye and complete loss of IOBs (Fig 2A and 2A'), a phenotype, which correlates with the expression of the site-specific CK2 phospho-variant, M8-S159D (see Chapter 2). We next tested the effects of M8- Δ MAPK and M8- Δ PD at the same stage. Unfortunately similar results did not bear out owing to embryonic lethality elicited by both the variants when expressed with *scaGal4*, even at 18C (as shown in Fig. 2 inset). To overcome lethality we used *109-68Gal4*, a weaker stage-2/3 driver. Expression of M8- Δ CK2 elicited a reduced eye and loss of the IOBs (data not shown), but not with the same severity as seen with *scaGal4*. The difference in severity of the reduced eye may be an effect of stronger strength of *scaGal4*, as compared to *109-68Gal4*. Both M8- Δ MAPK and M8- Δ PD elicit a strong reduction in eye size (Fig. 2B and 2C) and also display defects in IOB patterning (Fig. 2B' and 2C'). No eye/IOB defects are seen in *scaGal4/+* or *109-68Gal4/+* flies or the three M8-deletion lines on their own. These studies indicate that the MAPK motif is also modulated for regulating M8 activity. An intact P-domain is necessary to elicit autoinhibitory effects on the protein. In

absence of the P-domain, M8 can be precociously activated which then antagonizes Ato and perturbs R8 specification.

M8 deletion variants perturb R8 specification and bristle formation

In order to determine if the deletion variants perturb birth of R8 and secondary photoreceptors, we next stained the third instar larval eye disc with Senseless (Sens), a marker for differentiated R8s and ELAV- a pan-neuronal marker. The reduced eye upon expression of M8- Δ CK2 with *scaGal4* (Fig. 3A) reflects strong loss of R8s (Sens+ cells), and many of the R8s that emerge from the MF poorly sustain Sens expression, and thus inefficiently recruit secondary (ELAV+) photoreceptors. A few regions of the eye disc are devoid of closely juxtaposed ELAV clusters, as compared to control discs (data not shown). These R8s are likely removed by apoptosis, a default fate for uncommitted cells, contributing to the 'rough eye' of M8- Δ CK2 expressing flies (see Fig. 2A).

As expression of M8- Δ MAPK and M8- Δ PD with *scaGal4* was lethal, we used *109-68Gal4* to enable analysis of those two M8 variants. In contrast to M8- Δ CK2, discs expressing the M8- Δ MAPK and M8- Δ PD display the inconsistent maintenance of Sens levels along the AP axis in a greater proportion of R8s (Fig 3B, C). Moreover, significant regions of the eye disc altogether lack Sens+Elav clusters, a result consistent with the reduced eye (Fig. 2B, C). Thus the reduced eyes of the variants described in Fig. 2 reflect defective birth, delayed differentiation and survival of R8s, and impaired secondary photoreceptor recruitment. It is possible to deduce from the severity of R8 defects as seen from the three different deletion variants, that M8- Δ CK2 likely acts as a 'sink' for modification by other kinases, which is required for repressor activity of M8. As seen in R8 specification defect with M8- Δ PD, the need for those modifications are bypassed, which reflect in severe deformity of the R8 and secondary photoreceptor clusters.

In addition to the reduced eye, all three M8 deletion variants display a strong loss of MC's and mc's on the thoracic and scutellar regions. This reflects studies showing both *scaGal4* and *109-68Gal4* enhancer trap lines are active in the bristle PNC's. We quantified the MC loss in the heminotum region for all three-deletion variants. The severity of attenuation of MC's for M8- Δ CK2 with *scaGal4* (Fig. 3A') is comparable to the loss elicited by M8- Δ MAPK and M8- Δ PD when driven by *109-68Gal4* (Fig. 3B', 3C'). The loss of mc's, however, showed less severity when driven by *109-68Gal4* as compared to *scaGal4*. The lack of effects on the mc's reflects weaker strength of *109-68Gal4*. Together, these studies reveal that the P-domain is important for M8 activity in the eye as well as bristle formation.

The effects of M8- Δ CK2 are sensitive to altered EGFR levels

Studies described in Chapter 2 implicated the importance of MAPK site in regulating repressor activity of M8 during lateral inhibition. This raises the possibility that M8 may be a target of activated EGFR signaling during R8 specification. If that is the case, then it is expected that the retinal defect of the M8- Δ CK2 variant should be sensitive to altered EGFR dosage, whereas the M8-MAPK and M8- Δ PD variants should be refractory to changes in EGFR signaling as the MAPK site is already compromised in those two variants.

For this analysis, we used *egfr^{f24}*, a loss of function (amorphic) allele, which does not perturb the eye/R8s in the heterozygous state (data not shown) but is lethal when homozygous. If EGFR pathway indeed regulates the activity of M8, then a decrease in its level should attenuate the level of active MAPK, and result in hypo-phosphorylation of M8 at its MAPK site. As mentioned earlier, in such a condition (*egfr^{f24}/+* background) the reduced eye phenotype of M8- Δ CK2 should be suppressed. This prediction bears out as we see that the severity of reduced eye of M8- Δ CK2 in an *egfr^{f24}/+* background is indeed significantly attenuated when compared to its effects in *egfr+/+* background (Fig. 4A). This result is supported by the qualitative analysis of the eye disc, which shows a strong suppression of

the R8 defects in an *egfr^{f24}/+* background (Fig. 4A') in comparison to the strong loss of patterned R8s (Sens+ cells) as seen in *egfr+/+* background (Fig. 3A).

A similar analysis was conducted with the M8-MAPK and M8-ΔPD variants, but utilized the driver *109-68Gal4* due to lethality upon expression with *scaGal4* (see Fig. 2 inset). The severity of perturbation of eye defect due to overexpression of these two variants was indistinguishable in *egfr⁺* versus *egfr^{f24}/+* backgrounds (compare between Fig. 2B,C and Fig. 4B,C). Staining of the eye disc with Sens and ELAV display similar neural hypoplasia reflecting loss of founding R8's as seen in an *egfr+* background (compare Fig 3B,C and Fig. 4B',C'). Based on this data it can be hypothesized that the two closely clustered kinase sites, MAPK and CK2, may participate in 'cis'-inhibition of M8. Since this interaction is lost when the entire P-domain is deleted, the protein bypasses the necessary post-translational modifications and is presented as a prematurely activated repressor.

M8-CtD strongly suppresses the eye and bristle defects of all the deletion variants

It has been previously reported that co-expression of the 56 residue peptide from M8 (M8-CtD) rescue the reduced eye and bristle defect of M8-S159D (Kahali et al., 2010b). Besides recovery of the eye size, it was also shown that co-expression of M8-CtD significantly restored the hexagonal facet phasing and the position of the IOB's. Importantly, rescue does not involve competition between two *UAS*-constructs for a limiting amount of Gal4 protein, because no rescue of *109-68Gal4>M8-S159D* was evinced upon co-expression of *UAS-LacZ* (data not shown). We therefore tested for rescue of the reduced eye of all the deletion variants by co-expressing CtD peptide of M8. Indeed, co-expression of M8-CtD rescued the reduced eye of M8-ΔCK2, M8-ΔMAPK and M8-ΔPD (Fig.5 A, B, C). It also significantly restored the position of IOB's (data not shown). M8-CtD largely restored the eye field of M8-ΔCK2, almost close to the WT eye, whereas restoration of M8-ΔMAPK and M8-ΔPD was also significant.

In addition to the reduced eye, deletion variants also displayed strong loss of the four scutellar MC's (Fig. 3), because the enhancer traps, *scaGal4* and *109-68Gal4* are active in the bristle PNC's (Kahali et al., 2009a; Powell et al., 2004). We wanted to assess whether this neural defect is modulated by co-expression of CtD-peptide. Indeed, our result shows that co-expression of M8-CtD peptide restored ~2 scutellar MC's in each of the deletion variants (Fig. 5E). It therefore appears that co-expression of M8-CtD peptide can effectively suppresses both the eye and the bristle defects of the ectopic deletion variants. The possible explanation for such rescue may be the result of binding of the ectopic M8-CtD (in '*trans*') to the non-inhibited deletion variants and impairing repression.

P-domain in M8 may be a target for modulation by Slmb

Recently, a *Drosophila* protein-protein interaction screen has revealed Slmb to be an interacting partner of M8 (Guruharsha et al., 2012). Slmb, a subunit of the E-3 ubiquitin ligase, interact with phosphorylated Ser residues mark the protein for proteasomal degradation. Detailed sequence analysis of the CtD of M8 indicates that phosphorylated Ser residues at 154-159 may create a consensus for Slmb binding.

We thus tested if Slmb has any effect on the deletion variants by asking whether co-expression of *UAS-Slmb* modulates their reduced eye effect. The reduced eye of all the deletion variants upon co-expression of *UAS-Slmb* (Fig. 6A', B', C') appeared similar to that in its absence (compare with Fig. 6A, B, C). This result is supported by quantitative analysis of eye size (Fig. 6D). The eye sizes in the presence of ectopic Slmb were statistically similar to those in its absence. This suggests that an Slmb cannot target M8 in absence of its P-domain. The deletion of the CK2 motif takes out one of the target pSer residues for Slmb interaction, which thus fails to interact with the protein and cannot mark it for proteasomal degradation. Since, the pSer residue at the MAPK motif is not a target for Slmb binding, it was expected that co-expression of Slmb will rescue ectopic M8- Δ MAPK eye defect. But

our predictions did not bear out (compare Fig. 6B and 6B'). We think that dually phosphorylated M8-CtD (Ser¹⁵¹⁺¹⁵⁹) serve as a template for hierarchical modification of both Ser¹⁵⁴ and Ser¹⁵⁵, by CK1 and GSK3 respectively, forming a consensus for Slmb target. Since M8-ΔMAPK is missing Ser¹⁵¹, the following Ser residues cannot be phosphorylated, which then fails to generate Slmb phosphodegron. In summary, we think that an intact P-domain is essential for Slmb target of M8 and its subsequent degradation.

Discussion

The activities of E(spl) proteins are vital for mediating the effects of inhibitory Notch signaling via lateral inhibition. Therefore it is important to understand the underlying mechanism of regulation of E(spl) proteins during neural repression. Previous work from our lab has demonstrated that the CtD of M8 plays a definite role in autoinhibition of the protein to prevent its precocious activation (Kahali et al., 2010a). It has been shown that CK2 kinase phosphorylates a specific Ser residue in the CtD, which perhaps sets the protein in an active state when it can bind and antagonize Atonal during R8 specification (Karandikar et al., 2004). Further studies, as discussed in Chapter 2 suggest that phosphorylation of M8 by CK2 more likely acts as a conformational 'gatekeeper', and that modification by a second kinase, the MAPK acts as a final activating switch. In a sense, CK2 and EGFR together may therefore control when and where M8 activity manifests in the developing retina. The putative role of EGFR in regulating M8 activity was strengthened as our studies show that the phosphomimetic variants of M8 were responsive to altered EGFR levels (see Chapter 2). The above data compelled us to look closer and analyze the role of CtD in autoinhibition of M8 during neurogenesis. Sequence analysis of the CtD of M8 revealed invariant Ser residues upstream of the CK2 motif across all the twelve *Drosophila* species, that diverged ~ 50x 10⁶ years (myr) ago. The remarkably conserved stretch of Ser residues in the CtD has been aptly termed the P-domain (phosphorylation domain) as it may be targeted by other

kinases. The P-domain of M8, M5 and M7 are the only conserved region in the CtD, which is otherwise variable in length and sequence between the different members. A similar P-domain is also present in human HES6, which has been shown to be regulated by CK2 in a manner akin to M8 (Gratton et al., 2003a). Although the developmental consequences of phosphorylation of the other Ser residues in the P-domain of HES6 are presently unknown, it provides us a window of opportunity to investigate and understand the role of P-domain in autoinhibition.

The studies we present here directly test the role of P-domain in autoinhibition model in two relevant developmental contexts, the eye and the bristle. We have provided multiple lines of evidence that the P-domain, which has conserved Ser residues, undergoes modifications by different kinases to relieve autoinhibitory effects on M8. In addition, the phosphorylated P-domain is suspected to play a role in formation of 'phosphodegron', which is a necessary step for protein degradation and clearance.

Our first line of evidence that P-domain may be involved in repression of M8 comes from our deletion variant studies. If CtD autoinhibits M8 in a phosphorylation-dependent manner one might expect deletion of the CK2 motif should render M8 hyperactive. Indeed, M8- Δ CK2, which lacks just the CK2 consensus site (S¹⁵⁹DCD) (Fig.1), perturbs the eye, but only in a stage-specific manner. It elicits a reduced eye when expressed at stage-2/3 of the MF (Fig. 2), but fails to do so at stage-1 (Fig.1). The inactivity of M8- Δ CK2 at stage-1 reveals that this protein is not constitutively active, raising the possibility that it requires additional conformational modifications, which may occur at stage-2/3. The presence of a consensus MAPK site in the CtD could provide a means for full activation of M8 by EGFR/MAPK signaling at stage-2/3 of MF. To understand the role of the MAPK motif and the P-domain in autoinhibition, the deletion variants M8- Δ MAPK and M8- Δ PD were overexpressed in a similar manner at stage-1 and stage-2/3 of the MF. The MAPK and P-

domain deletion variants both perturb the eye at stage-2/3 of the MF (Fig. 2). These are in accordance with the theory that due to the deletion of the kinase-specific motifs, the proteins can by-pass the need for post-translation modification and act as a repressor and suppress Ato immediately. The overexpression of M8- Δ MAPK variant also elicits eye defect at stage-1 of the MF. The most parsimonious interpretation for such a result is that perhaps M8- Δ MAPK, which is already in a hyperactive state, can precociously repress Ato, even at stage-1 of the MF (Fig. 1) where Ato levels are lowest. If the mechanism of autoinhibition is dependent on phosphorylation then overexpression of M8- Δ PD at stage-1 should have elicited more severe eye defect. Unfortunately, analysis of M8- Δ PD at stage-1 did not bear out owing to its embryonic lethality (Fig. 1).

Secondly, the rescue of the severely reduced eye of the M8 deletion variants by CtD-peptide, an ectopically expressed subdomain of M8, points to the fact that indeed the P-domain is part of the contact-dependent regulatory mechanism between the HLH and/Orange domain and the CtD of the protein. It would therefore seem to be the case that the ectopically expressed CtD peptide binds (in '*trans*') to the non-inhibited deletion variants and attenuates their interaction with Ato. Consequently, Ato level would rise to a specific threshold to confer the R8 fate, which would increase eye size (facet numbers), as seen in our result (Fig. 5).

Additional role of P-domain in M8 degradation can be speculated from Slmb overexpression studies. The region between the MAPK and CK2 consensus motifs has two conserved Ser residues. A bioinformatics study revealed that these two residues could be targeted for modifications by CK1 and GSK3. A large -scale *Drosophila* protein-protein interaction in S2 cells revealed M8 as an interaction partner with Slmb (Guruharsha et al., 2012). Hence, it is of interest to note that phosphorylation of the additional Ser residues is predicted to generate a strong site for Slmb/ β -TrCP binding, which can then promote

proteasomal degradation for M8, providing a possible mechanism for clearance of M8 from the MF. A study in our lab revealed that co-expression of Slmb can rescue the reduced eye phenotype of ectopic M8-S159D, a CK2 mimic of M8 (Majot and Bidwai, personal communication). The most parsimonious explanation is, since the additional Ser residues in the P-domain of M8-S159D are intact, they can undergo modifications by CK1 and GSK3, generating the Slmb phosphodegron, leading to the subsequent removal and clearance of ectopic M8-S159D. If this same logic were applied in case of the deletion variants, one would think that the perturbation of eye displayed by M8- Δ PD, which does not have the target Ser residues for Slmb binding, will not be rescued by Slmb overexpression. Indeed that is the case. The eye defects of M8- Δ PD cannot be modulated by co-expression of Slmb (Fig. 6). In addition we see similar results with M8- Δ CK2 and M8- Δ MAPK. This would suggest that a hierarchical phosphorylation is needed to generate a putative Slmb-binding site and since both these deletion variants are missing one of the consensus motifs for phosphorylation, it may disrupt subsequent modifications of the remaining Ser residues, leading to impairment of Slmb binding and generation of a stable phosphodegron.

Our studies indicate that the P-domain in M8 has two modes of action. The first one is to autoinhibit bHLH and/Orange domain through intramolecular binding, thus preventing inappropriate association with Ato. This theory is in concordance with the activity of M8*, a protein which lacks the CtD and elicits eye defect when ectopically expressed. Similar eye defects are not mimicked by expression of full length M8, which strongly suggest that the truncation triggers the dominant behavior. Thus, it is imperative that M8 must have an intact P-domain to elicit autoinhibitory effects. Second mode of action is to provide a mechanism for rapid clearance of the protein after it has repressed Ato during lateral inhibition. Based on the various modifications the P-domain in CtD undergoes, an epistatic model for regulation of M8 through multisite phosphorylation has been suggested (Fig. 8). As

mentioned earlier, dual phosphorylation of M8 by CK2 and MAPK sets the protein in an active state, which can then repress Ato. Phosphorylation of the residues 159 and 151 by CK2 and MAPK respectively, may prime M8 for progressive phosphorylation at residues 154 and 155. Previous work from our lab has suggested that phosphatase PP2A can dephosphorylate M8 at Ser 151 and controls rapid inactivation of the protein (Bose et al., 2014). Slmb can target the other three phosphorylated residues in P-domain (154, 155 and 159) facilitating ubiquitinylation and proteasomal degradation (Fig. 8). This tandem employment of PP2A and Slmb provides a mechanism for rapid reduction in E(spl) activity. Endogenous M8 is non-functional at stage-4 of the MF as R8 specification is complete by then. If so, the deletion variants should have no effect at stage-4 of the MF. But our studies reveal otherwise. The deletion variants effect eye development even at stage-4 of the MF (Fig. 7). It is possible that the deletion variants lacking the consensus Ser residues for formation of Slmb phosphodegon are not targeted for proteasomal degradation and subsequent rapid clearance and thus remain in the MF even after their inhibitory effects during R8 specification has been realized. It is quite likely that these ectopic deletion variants are targeting other proteins in the secondary photoreceptor specification pathway and eliciting perturbation of the eye, as we see in our data. This mode of control via phosphorylation followed by rapid inactivation and/or clearance provides a mechanism for control of M8 activity levels during R8 specification.

Future studies to identify the site on the HLH or Orange motif/residues mediating this interaction with P-domain will be required to fully reveal the mechanism(s) of regulation of M8 during neural repression. The relevance of this mechanism to other E(spl) members will define the 'qualitative' difference between them and also help understand the mechanisms underlying repression by this group of bHLH proteins.

Materials and Methods

Construction of M8 deletion variants: Three M8 deletion variants were generated by inverse-PCR. M8-ΔCK2 variant harbors the deletion of the CK2 motif (S159DCD), M8-ΔMAPK variant is missing the MAPK (P149LSP) motif and M8-ΔPD variant is missing the entire P-domain (P149LSPASSGYHSDCD). All the three deletion constructs were amplified by PCR using custom forward and the reverse primers incorporating EcoRI and BamHI sites 5' and 3' to the coding region, respectively. The forward primer incorporated a KOZAK consensus (CAAC) immediately 5' to the ATG codon for efficient expression. All constructs were fully sequenced to confirm the presence of only the intended mutations.

Germ line transformation: For *in vivo* expression, all the three deletion constructs were cloned into pUAST. Transgenic lines were generated using a commercial embryo injection facility (BestGene, Inc.). w+ progeny were identified and the location of the insertion sites for the different variants were mapped by standard approaches using chromosomes harboring dominant visible markers. At least 10 independent insertions of M8-ΔCK2, M8-ΔMAPK and M8-ΔPD were generated, and of these ≥5 have been used in the studies.

Biochemical analysis: Protein-protein interactions were analyzed by the LexA-based version of the yeast interaction trap (Gyuris et al., 1993). The bait and prey constructs were expressed as C-terminal fusions with DNA-binding domain of LexA and the activation domain (AD) of B42, respectively. The yeast strain EGY048p was used to evaluate protein interactions, as described (Trott et al., 2001). LacZ activity was determined for at least three independent transformants, each in triplicate.

Fly stocks: The Gal4 drivers were obtained from Bloomington Stock Center (denoted by the prefix B). These drivers are *109.68Gal4* (B6479), *h^{H10} Gal4* (Huang and Fischer-Vize, 1996), *scaGal4* (Giebel and Campos-Ortega, 1997). The DER mutant line *egfr^{f24}* (B6500)

was also obtained from Bloomington Stock Center. UAS-*Slmb* flies were obtained from Bloomington Stock Center.

Eye and bristle phenotypes: All crosses were performed on standard Yeast-Glucose medium at 24°C, unless indicated otherwise. Fly heads were dehydrated by sequential passage through a graded alcohol series for 24 hours each (25%-50%-75%-100%), and finally passed through Hexamethyldisilizane. Heads were mounted on EM stubs, dried for 24 hours, sputter coated with gold and examined with a JEOL-6400 scanning electron microscope at an accelerating voltage of 10-20kV. Images were processed with Adobe Photoshop and collated in Adobe Illustrator. The reduced eye was quantified from ~20 adult flies that were photographed using a Leica MZ16 stereomicroscope equipped with a Leica DFC-480 digital camera. Facet numbers were counted from TIFF images. A similar approach was used to determine bristle phenotypes. Eye size (facet counts) and bristle defects were statistically analyzed using Student's T-test.

Immunostaining and confocal microscopy: Eye imaginal discs were isolated from late third instar larvae and processed as described (Kavler et al., 1999) with minor modifications. After isolation, discs were fixed in 4% paraformaldehyde in 1x phosphate buffered saline (PBS) for 45 minutes at 4°C. After fixation, discs were washed three times with PBS containing 0.3% Triton X-100 (PBS-TX) for 15 minutes each. The discs were incubated for 12-14 hours at 4°C in PBS-TX containing primary antibody. The following antibodies were used; guinea pig anti-Sens (gift from Hugo Bellen, HHMI-Baylor) at a dilution of 1:2000 and mouse anti-ELAV (DSHB, Iowa) at a dilution of 1:1000. Following primary antibody binding, eye discs were washed three times with PBS containing 0.3% Triton X-100 and then incubated with secondary antibody at room temperature for 2-3 hours. The secondary antibodies (Molecular Probes) used for are, goat anti-guinea pig-IgG coupled to Alexa Fluor 633 (1:1000) and goat anti-mouse-IgG coupled to Alexa Fluor 488 (1:1000). The discs were

mounted in 60% glycerol, and viewed on an Olympus FluoView (FV100) for confocal imaging. Images were acquired every 1 μ m along the apicobasal axis of the discs and then compressed as a Z-stack without the removal of any layers. Compressed Z-stacks were exported as TIFF files and collated in Adobe Illustrator.

Fig. 20: Deletion variants and their MF specificity.

(A) The functional domains of E(spl)-M8 are represented by a basic domain (DNA binding), an HLH domain (dimerization), a second HLH region, the Orange domain (interaction with Ato/ASC) and a C-terminal WRPW motif (Gro-binding). Schematic representation of the random deletion mutations of M8 used for the study. M8-ΔCK2 lacks the CK2 motif, M8-ΔMAPK lacks the MAPK motif and M8-ΔPD lacks the entire P-domain. (B) Two-hybrid interaction of the M8 deletion variants with Gro (co-repressor) was measured as Lac Z activity. (C) Overexpression of M8-ΔCK2 fails to generate any eye defect at stage-1 of the MF. (D) M8-ΔMAPK perturbs the eye at stage-1 of the MF. Note that any study at this stage with M8-ΔPD was precluded owing to embryonic lethality (EL). (C',D')1000x magnification of the corresponding genotypes in panels (C-D). (E) Ommatidial (facet) counts were determined in ≥ 25 of the indicated genotypes. Asterisk denotes P-value < 0.001 .

Fig. 21: Deletion variants enhance M8 activity at stage-2/3 of MF.

(A-C) Adult eye phenotypes of different M8 deletion variants at stage-2/3 of the MF. Overexpression of M8-ΔCK2 with *scaGal4* perturbs the eye. Note that overexpression of M8-ΔMAPK and M8-ΔPD with *scaGal4* leads to EL (embryonic lethality) owing to which the study was performed with *109-68Gal4*. Magnification is at 200x. (A'-C') 1000x magnification of the corresponding genotypes in panels (A-C). There are extra no IOBs for specified for M8-ΔCK2 (A'), whereas, both M8-ΔMAPK (B') and M8-ΔPD (C') shows missing as well as extra IOBs. (D) Ommatidial (facet) counts were determined in ≥ 25 of the indicated genotypes.

Fig. 22: Perturbation of R8 birth and differentiation and bristle formation at stage-2/3 of MF.

Eye disc of the aforementioned genotypes were immunostained with α -Sens (magenta) and α -ELAV (green) to determine R8 differentiation and secondary photoreceptor recruitment respectively (A-C). Arrow indicates direction of MF progression. Note that all three-deletion variants show random patches of non-specified and non-differentiated retinal tissue at stage-2/3 of the MF. (A'-C') The deletion variants elicit loss of macrochaetes (MC's) and microchaetes (mc's) with similar potency. These effects on the MC's/mc's reflect expression of *scaGal4/109-68Gal4* in proneural clusters that give rise to these bristle types.

Fig. 23: Decreased level of EGFR rescue reduced eye of *M8-ΔCK2* only.

(A) Overexpression of *M8-ΔCK2* at *egfr^{f24/+}* background restores the eye field. Overexpression of (B) *M8-ΔMAPK* and (C) *M8-ΔPD* at stage-2/3 of the MF at *egfr^{f24/+}* background fails to restore the eye field. (A'-C') Eye discs of genotypes indicated in A, B and C were immunostained with α -Sens (magenta) and α -ELAV (green) to assess R8 differentiation and secondary photoreceptor recruitment. Facet count was determined in

≥25 flies. Data labeled A, B and C corresponds to the adult eyes shown in panel (A-C). Asterisk denotes P-values < 0.001. Note that the data was compared to eye facet count of the over-expression study adapted from Fig. 2.

Fig. 24: M8-CtD suppresses the eye and bristle defects of deletion variants.

(A) Schematic representation of the functional domains of M8 and M8-CtD. The C-terminal domain (CtD) peptide encompasses residues 123-179 of full length M8 and harbors the entire P-domain with intact MAPK and CK2 phosphorylation sites along with the terminal Gro-binding site. (B) Rescue of the reduced eye of *scaGal4 > M8-ΔCK2* upon co-expression of the CtD. Magnification is at 200x. (C-D) Rescue of the reduced eye of *109-68Gal4 > M8-MAPK* and *109-68Gal4 > M8-PD* upon co-expression of the CtD. (E) Ommatidia facet counts were determined in ≥ 25 of the indicated genotypes. (F) Suppression of the MC defects of the different genotypes upon co-expression of the CtDs. Numbers to the right denote the average scutellar MC count in corresponding number of flies.

Fig. 25: P-domain in M8 may be a target for modulation by Slmb.

Scanning EM of the adult eye at 200x magnification (A-C). Reduced eye phenotypes of the deletion variants at stage-2/3 of the MF. (A'-C') Co-expression of Slmb fails to rescue the reduced eye phenotype due to overexpression of (A) *M8-ΔCK2*, (B) *M8-ΔMAPK* and (C)

M8- Δ PD. Graph shows ommatidial (facet) counts of the adult eyes. Facet counts were determined in ≥ 20 flies, and asterisk denotes P-values < 0.001 .

Fig. 26: Deletion variants perturb the eye at stage-4 of the MF.

Scanning EM of the adult eye at 200x magnification. The adult eye of the deletion variants expressed at stage-4 of the MF with *gmrGal4*. (A) The *M8-ΔCK2* variant shows a rough eye phenotype whereas *M8-ΔMAPK* (B) and *M8-ΔPD* (C) both shows severely reduced eye. Graph shows ommatidial (facet) counts of the adult eyes. Facet counts were determined in ≥ 20 flies. (A'-C') 1000x magnification of the corresponding genotypes in panels (A-C). All

three variants show 'blueberry' phenotype represented by 'sunken' ommatidia. M8- Δ CK2 (A') shows a more muted phenotype as compared to M8- Δ MAPK (B') and M8- Δ PD (C') shown by dotted circles. In panel (A') the arrow represents fused ommatidia.

Fig. 27: Epistatic model of M8 activity in the MF.

Model shows the P-domain of M8 with conserved Ser residues (in green). The four Ser residues are phosphorylated in an epistatic manner. The Slmb protein recognizes the phosphorylated Ser residues at 154, 155 and 159. Inset shows consensus motif for Slmb recognition. The schematic representation of different states of M8 has been shown. At autoinhibited state, P-domain blocks HLH and/Orange domain. MAPK and CK2 phosphorylation sets the protein at an active state, followed by additional modulation by CK1 and GSK3. M8 can then be rapidly inactivated by dephosphorylation by PP2A and finally targeted for proteasomal degradation by formation of Slmb phosphodegron.

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