GENETIC MECHANISMS INVOLVED IN AXIAL PATTERNING AND NEURODEGENERATION IN DROSOPHILA EYE

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GENETIC MECHANISMS INVOLVED IN AXIAL PATTERNING AND NEURODEGENERATION IN DROSOPHILA EYE

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ABSTRACT

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Complex network of genetic and molecular mechanisms governing the process of organogenesis have an important bearing on development of organisms. We are using an established model of Drosophila melanogaster commonly referred to as fruit fly in order to understand these mechanisms. We have used Drosophila eye to discern genetic hierarchy controlling the (i) event of axial patterning, and (ii) to study neurodegeneration in the developing eye. Axial patterning involves generation of dorsal-ventral (DV), anterior-posterior (AP) and proximal-distal (PD) axes in the organ primordium and is considered crucial for transformation of monolayer epithelium into a three dimensional organ. Any abnormalities in expression patterns of axial patterning genes may result in complete loss of organ. Drosophila eye develops from a default ventral state conferred by expression of genes Lobe (L) and Serrate (Ser). It has been found that antagonistic interaction of dorsal and ventral genes helps generation of midline or the equator which is essential for growth and differentiation of the eye field. Loss-of-function of L/Ser results in
complete or loss-of-ventral eye depending on time axis involved. In a genetic modifier screen performed for search for modifiers of $L$ mutant phenotypes, an E3 ubiquitin ligase, Cullin-4 (Cul-4) and GATA-1 transcription factor Pannier (Pnr) were identified. In the current study, we have characterized Cul-4, in promoting cell survival in the ventral domain of developing eye via downregulation of Wingless (Wg) signaling. Cul-4 also regulates JNK signaling to prevent cell death in the developing eye. We thus place the Cul-4 in the hierarchy of ventral genes involved in eye development.

We also present the role of GATA-1 transcription factor Pnr in defining the dorsal eye margin boundary by suppressing the eye fate. $Pnr$ downregulates retinal determination gene machinery via zinc finger transcription factor teashirt ($tsh$). We thus provide a novel mechanism involved in defining dorsal margins of the eye during early stages of organogenesis and an eye suppression function, as a late role of $pnr$ in the developing eye.

Identification and characterization of these genes in the dorsal and ventral domains of the eye may help enrich our understanding of the genetic hierarchy and the complex interactions of genes involved in axial patterning in the eye during organogenesis. Since the genetic machinery is highly conserved from flies to humans, these studies will have direct implications on higher vertebrates as well.

Other than patterning and growth studies, Drosophila eye has been widely used to study genetic and molecular basis of neurodegeneration. A part of current study is to test the mechanisms involved in the neuronal cell death caused during the course of Alzheimer’s disease (AD). AD is caused due to accumulation of Aβ-42 peptide which is a product formed because of incorrect cleavage of Amyloid Precursor Protein (APP). Accumulation of Aβ-42 results in formation of amyloid plaques which eventually results into stress and the neuronal cell death. We
have found that JNK signaling pathway is induced upon Aβ-42 accumulation and causes cell death of the neurons in the brain. Our study provides a new mechanistic insight from the perspective of identifying the new targets of AD neuropathy.
Dedicated to my Mother....
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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

A. Introduction to Drosophila

*Drosophila melanogaster*, a.k.a. fruit fly has emerged as the “Golden Bug” over the past century (Bier, 2005). The ease and robustness of culture, short life cycle and the convenience to screen large sample sizes for experiments makes them superior to other available genetic model systems. *Drosophila* has become a highly versatile and sophisticated tool for carrying out genome-wide genetic screens. First reported as a model organism by embryologist T.H. Morgan in 1908, this golden bug has evolved as a handy tool for geneticists and developmental biologists.

The entire *Drosophila melanogaster* genome is sequenced and there is a higher degree of conservation between flies and humans. Therefore, *Drosophila* has not only been used as an ideal tool for the genetic analysis, but can also be used to study numerous human diseases including cancer, neurodegenerative disorders, developmental diseases, cardiovascular diseases, metabolic disorders and many more (Bier, 2005).

*Drosophila* genome is comprised of approximately 13,600 genes (Adams et al., 2000) and contained in four chromosomes: First or Sex chromosome (1), Second (2); divided into left (2L)
and right arms (2R), Third (3); divided in left (3L) and right arms (3R); and the short Fourth (4) chromosome. Thus, first chromosome is sex chromosome while 2, 3, 4 are autosomes.

*Drosophila melanogaster* is a holometabolous insect, (Johnson and Triplehorn, 2004) with a high reproducible capability. The life cycle of fly consists of several different larval instar and pupal stages. A healthy adult female can produce 300 progenies in her life time. The female has special sperm storage organs (the spermatheca and seminal receptacle) that allow her to produce several hundred progeny after a single mating (Price et al., 1999). The embryo gastrulates to generate embryonic germ layers including ectoderm, endoderm and mesoderm and completes the development in less than 24 hours. The end of embryogenesis is marked by hatching of worm-like first instar larva from the egg (Hartwell, et al, 2011).

The first instar larva eventually grows in size during the second (24 hours after hatching) and third instars larval stages (48 hours after hatching). The third instar larva pupates under the pulse of steroid hormone ecdysone and metamorphoses into an adult fly. The first second and third instar stages (termed as L1, L2 and L3 stages) harbor all the organs as organ primordia, referred to as imaginal discs (Fig. 1.2). Imaginal discs are flattened sac-like structures which are set aside during the embryonic stages. These discs undergo extensive growth and differentiation during the larval and pupal stages. At the end of metamorphosis, when adult is ready to emerge from the pupal case, wings expand and exoskeleton is hardened and becomes pigmented (Hartwell, et al, 2011).

Another important aspect that makes fly a superior model is the availability of vast variety of tools to manipulate the function of different genes at different time points as required throughout the life cycle. There are tools available which aid in manipulating gene function from embryonic stages to pupal stages in the desired tissues in a targeted fashion. These tools can be used to reduce the gene functions (RNAi approaches), abolish the gene function (targeted knock
outs) or even misexpress the particular gene (targeted over expressions). Genetic mosaic techniques are widely used to induce genetic changes in a subset of cells or tissues in an individual organism in order to study function of an embryonic lethal gene. The convenience of using these genetic mosaics in *Drosophila* makes it an ideal model for performing spatio-temporal analysis of any gene. The tools used in our study have been described in details in the Materials and Methods section.
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B. Review of Literature

A glimpse into Dorso-ventral patterning of the *Drosophila* eye

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INTRODUCTION

During development, the patterning signals progressively restrict cell fates by subdividing a large developing field into smaller fields with limited developmental potential. These smaller fields that correspond to the domains of expression of selector genes are referred to as the compartments. The selective spatio-temporal expression pattern of the cell fate selector genes is responsible for the formation of compartments (Blair, 2001; Curtiss et al., 2002; Held, 2002; Dahmann et al., 2011). The boundary between the compartments, where two different cell types are juxtaposed, is responsible for generating new signaling centers to regulate patterning, growth and differentiation of a developing field (Meinhardt, 1983; Blair, 2001). Thus, formation of the developmental boundaries is crucial for maintaining the downstream patterning events (Blair, 2001; Curtiss et al., 2002; Dahmann et al., 2011). Therefore, an important question in developmental biology is how these boundaries are generated and maintained during development. The aim of this review is to provide an overview of recent advances on generation of the boundary between the dorsal and ventral compartment of the eye and its implications on development of the eye as an organ. This process is referred to as Dorso-Ventral (DV) patterning of the Drosophila eye. The Drosophila eye, an ideal model system for studying organogenesis, has been extensively used to investigate tissue patterning and cell-cell communication during axial patterning. Furthermore, Drosophila eye serves as an excellent model to understand the genetic mechanism responsible for division of a developing field into several smaller fields with positional fate restrictions (Singh et al., 2005b). The genetic machinery that controls Drosophila eye development closely resembles that of higher vertebrates suggesting conservation of certain genetic pathways throughout evolution
Axial patterning, which is essential for organogenesis in all the multi-cellular organisms, involves formation of Antero-Posterior (AP), Dorso-Ventral (DV) and Proximo-Distal (PD) compartments (Cohen et al., 1993; Cohen, 1993). During axial patterning of the wing and the leg imaginal discs, the sequence of events involves first the division of a field into anterior and posterior compartments of independent cell lineages, followed by subdivision of these imaginal discs into dorsal and ventral compartments. Interestingly, this sequence of division is not followed in the developing eye imaginal disc because it does not have analogous anterior-posterior axis. Instead, DV patterning is the first lineage restriction in the developing eye imaginal disc (Singh and Choi, 2003; Singh et al., 2005b). Despite the differences in the sequence of events, evidence suggests that some aspects of DV patterning mechanism are highly conserved in the developing eye and the wing. An important common conclusion is that the border between DV compartments is a center for organizing the growth and patterning of the disc. In the subsequent sections of the review, we will focus on the mechanism of generation of DV domains in the developing eye, and the genetic basis for the establishment of the DV pattern.

2. Eye imaginal disc

In *Drosophila*, a holometabolous insect, the primordia of all the adult structures are sequestered in the larva as epidermal invaginations that are called imaginal discs (Bodentstein, 1950; Ferris, 1950; Atkins and Mardon, 2009). The adult eye, antenna, head cuticle and head structures develop from a common developing field called as eye-antennal imaginal disc (Cohen, 1993; Held, 2002). The regions of the eye-antennal imaginal disc, which give rise to head structures including ptilinum, frons and maxillary palpus, originate from five embryonic
segments and the acron (Jurgens and Hartenstein, 1993; Younossi-Hartenstein and Hartenstein, 1993). The monolayer epithelium does not accurately reflect the sac like anatomy of the imaginal discs (Gibson and Schubiger, 2001). *Drosophila* imaginal disc are contiguous cell sheet of flattened epithelial cells with two opposing surfaces, a columnar epithelium called as disc proper (DP) and a squamous peripodial epithelium called as peripodial membrane (PM) (McClure and Schubiger, 2005; Atkins and Mardon, 2009). The *Drosophila* retina develops from the DP while the PM of the eye-antennal disc gives rise to the adult head structures (Figure 1.3; Milner et al., 1983; Haynie and Bryant, 1986; Atkins and Mardon, 2009). The eye-antennal imaginal disc emerges from an embryonic imaginal primordium, which is an anterior-dorsal sac comprising of approximately 20 cells that are set aside during mid embryogenesis (Poulson, 1950; Garcia-Bellido and Merriam, 1969; Yamamoto, 1996). The embryonic precursors for imaginal discs grow asynchronously from rest of the developing embryo (Anderson, 1972b; Anderson, 1972a; Crick and Lawrence, 1975; Cohen, 1993; Held, 2002; Kumar, 2011). In first two larval stages, the eye imaginal disc cells divide and grow. The distinction between the developing antenna and the eye field begins to appear during the second instar larvae (Kumar and Moses, 2001; Kenyon et al., 2003; Dominguez and Casares, 2005; Atkins and Mardon, 2009). The developing eye field is further divided into precursors for the eye proper, head cuticle and the ocelli while the antennal field divides into precursors for the antenna and head cuticle. Retinal differentiation begins from the posterior margin of the eye imaginal disc during late second instar or early third instar stage of larval development (Ready et al., 1976). Since the retinal differentiation is synchronous in nature, it appears like a wave of differentiation initiating at the posterior margin of eye imaginal disc, which then proceeds anteriorly. The wave of differentiation is referred to as the morphogenetic furrow (MF, Figure 1.3 A, A’ arrowhead); it results in transition of an undifferentiated epithelium to differentiated cell types comprising of regularly spaced photoreceptor clusters (Ready et al., 1976; Wolff and Ready, 1993). Posterior to the furrow,
photoreceptor clusters are generated by a sequence of events including the selection of the R8 founder neuron and recruitments of additional photoreceptor precursors in the order of R2/5, R3/4 and R1/6/7 (Wolff and Ready, 1993; Kumar, 2011). Thus, if the eye imaginal disc is largely undifferentiated till second instar of development, an interesting question that arises is how compartments are identified in *Drosophila* eye imaginal disc.

3. **Dorsal and ventral compartments and the equator**

The adult compound eye comprises of approximately 800 unit eyes or ommatidia (Figure 1.3D). Each ommatidium consists of eight photoreceptor neurons assembled in an asymmetric trapezoidal pattern and when viewed from the top it resembles a honeycomb-like hexagonal facet. The surrounding cell types are non-neuronal and include pigment, cone cells and mechanosensory bristles (Figure 1.3E, F; Wolff and Ready, 1993). This pattern of organization is repeated in all ommatidia of the eye. However, despite the similarity in the cellular composition of each ommatidium, the spatial arrangement of ommatidia in the eye is organized in two orientations (Figure 1.3E, F). This orientation also serves as a marker to distinguish the dorsal and the ventral halves of adult eye. The photoreceptors are arranged in a trapezoidal fashion within an ommatidium. The ommatidial clusters within an eye are organized in a mirror asymmetry as they are polarized in the opposite directions (Figure 1.3E, F). The boundary between the ommatidia of the dorsal half and their mirror image ventral ommatidia is referred to as an equator (Figure 1.3E, F). This mirror symmetry which corresponds to DV axis or compartments of the adult eye have been described in many insect eyes (Dietrich, 1909). Since the developmental mechanisms underlying the DV pattern have not been studied in detail, it raises an interesting question of how the dorsal and ventral pattern is established.

The pioneering studies to discern the relation between the equator and the DV compartmental boundary in the *Drosophila* eye suggested that the equator is not determined as
the boundary between the dorsal and ventral cell lineages (Ready et al., 1976). Even though, the result from this study does not exclude the possibility that the dorsal and the ventral domains of the eye derive from two independent cell lineages, the lineage boundary may not precisely correspond to the equator. A series of elegant genetic analyses experiments involving a large number of mosaic clones in the adult eye and the head supported this idea (Baker, 1978). These experiments demonstrated that clones strictly follow the DV boundary, and do not intermingle near the DV border (Held, 2002). These results validated the hypothesis that *Drosophila* eye derives from DV compartments. The wing imaginal disc is divided into anterior and posterior groups of cells in its early stage of development, which is followed by further partitioning into dorsal and ventral compartments (Lawrence and Morata, 1976). To analyze whether the eye and the head are also subdivided into different domains by sequential compartmentalization as in the wing, another mosaic analysis was carried out. Nearly all clones (96%) were restricted to either dorsal or ventral domain of the eye thereby conforming to presence of a boundary between the dorsal and ventral cells. Thus, clones generated in the dorsal or ventral compartment did not cross the DV boundary. A few clones (4%) do cross the DV border, which is probably due to the fact that such clones might have been induced prior to compartmentalization (formation of dorsal and ventral compartment boundary) or two independent dorsal and ventral clones might have juxtaposed at the equator region thereby giving a false notion of a single clone not respecting the DV boundary (Baker, 1978). The DV lineage restriction observed in the adult eye was also confirmed in the developing eye imaginal disc where large clones do not cross the DV midline and showed sharp outline along the DV midline and the clones located within the dorsal or ventral domain had wiggly borders (Dominguez and de Celis, 1998). Unlike the DV lineage restriction that is established in the first instar larval eye imaginal disc; there are evidences that anterior-posterior restriction occurs a little later in the second instar eye imaginal disc. Thus, the significance of this anterior-posterior restriction remains to be seen as the morphological
distinction between the anterior and posterior regions. In the subsequent sections we discuss the role of DV patterning genes, and the generation of pattern along the DV axis of the eye.

4. Genesis of the eye

The DV boundary has been suggested as the site for the activation of Notch (N) signaling in the eye imaginal disc to promote growth (de Celis et al., 1996; Go et al., 1998; Baonza and Garcia-Bellido, 2000). However, if DV patterning occurs as late as seen in the adult eye based on the orientation of the photoreceptors, then it may not be crucial for the growth of the eye. Thus, efforts were channeled towards investigating whether DV patterning takes place earlier during eye development. The seminal papers from three different groups established that DV lineage restriction takes place earlier during larval eye development due to domain specific expression of the DV patterning genes (Cho and Choi, 1998; Dominguez and de Celis, 1998; Papayannopoulos et al., 1998). The DV patterning genes are a class of genes involved in generating and maintaining the DV lineage in the eye. These reports identified a new time line for the initiation of DV patterning to early larval development. They also identified the genes whose expression and/ or function was restricted either to the dorsal or ventral compartment of the eye. Thus, it was proposed that DV patterning in the eye is generated by the domain-restricted expression of the dorsal and the ventral eye genes (Cho and Choi, 1998; Dominguez and de Celis, 1998; Papayannopoulos et al., 1998; Cho et al., 2000).

The basic question was what is the default state of the early eye primordium? To answer this question, several groups directed their efforts to identify the genes that are expressed in the early eye primordium. During embryonic development the eye primordium begins as a homogenous group of cells that continue to grow during first larval instar to form the eye imaginal disc. All the cells of the first instar eye imaginal disc uniformly express Lobe (L), a gene known to be involved in ventral eye development (Singh and Choi, 2003). These studies revealed
that till the late first instar of larval eye development the entire eye primordium is ventral in fate and depends on the function of ventral genes like *L* and its downstream target *Serrate (Ser)* (Singh and Choi, 2003; Singh et al., 2005b; Kumar, 2011). Loss-of-function of the *L* gene, which is expressed ubiquitously in eye imaginal disc (Figure 1.4A), results in selective growth defects in the ventral half of the eye (Figure 1.4, 1.5B, C). The loss-of-function studies suggested that the requirement of *L* function evolves along the temporal axis (Singh and Choi, 2003; Singh et al., 2005b). During early eye development, loss-of-function of *L* results in the complete loss of eye field (Figure 1.3B). However, loss of *L* gene function later during eye development causes selective loss of ventral half of eye (Singh et al., 2005b). Loss-of-function of *Ser* also results in the similar loss of ventral eye phenotype (Table 1.1; Singh and Choi, 2003; Singh et al., 2005b). Interestingly, the timing of restriction of *L/Ser* functional domain from the entire developing eye field (Figure 1.4D, E) to only the ventral half of eye (Figure 1.4B, C) corresponds to the onset of *pannier (pnr)* gene expression along the dorsal margin of the eye (Table 1.1; Figure 1.6). During late first instar of eye development, the entire homogenous population of the ventral cells of the eye primordium transition into two distinct dorsal and ventral lineages with the onset of *pnr* expression on the dorsal eye margin (Singh and Choi, 2003). This suggests that the ventral fate is the ground state of the larval eye imaginal disc, and *L* and *Ser* are essential for survival and/or maintenance of this ventral state (Singh and Choi, 2003; Singh et al., 2005b; Singh et al., 2006).

5. **DV patterning in the eye**

5.1 **Genes regulating ventral eye growth**

*L*, a gene involved in ventral eye development (Table 1.1) was first reported in 1925, as a gene required for eye growth (Morgan et al., 1925) and was cloned in 2002 (Chern and Choi, 2002). *L* encodes an ortholog of PRAS40 (Oshiro et al., 2007; Vander Haar et al., 2007; Wang and Huang, 2009), and is required during all stages of larval eye development (Chern and Choi,
L protein is expressed in both dorsal and ventral domains throughout eye imaginal disc development (Figure 1.5A). Even though L is expressed uniformly in the entire eye field, L function is not required for growth and differentiation in the dorsal region of the eye (Chern and Choi, 2002; Singh et al., 2005b). Evidence for this ‘domain-specific’ function of L (ventral specificity) came from genetic mosaic analysis using a null mutation (Chern and Choi, 2002; Singh and Choi, 2003). Loss-of-function clones of L show a eye suppression phenotype specifically in the ventral eye, however the dorsal clones do not suppress eye fate and exhibit well-organized photoreceptor clusters (Chern and Choi, 2002; Singh and Choi, 2003). Further genetic analysis revealed that this domain specific L function in growth was downstream to N-signaling, mediating N function either in the same or parallel pathway (Chern and Choi, 2002). Thus, the growth of early eye disc is controlled asymmetrically in the dorsal and ventral domains.

Expression of L in both dorsal and ventral domains is puzzling since its function is only required for growth of the ventral domain. It is possible that an unidentified partner that is expressed in dorsal domain of the eye imaginal disc antagonizes the L function or L may be selectively activated by some yet to be identified partner in the ventral domain.

Another candidate gene that may be contributing to ventral eye development is *decapentaplegic (dpp)*, a member of TGF-β family of proteins, which acts as a long range secreted morphogen (Table 1.1; Nellen et al., 1996; Chanut and Heberlein, 1997). Dpp forms a gradient in the early eye anlage (anterior brain and eye field) that transverses from dorsal to ventral (Chang et al., 2001). In early eye imaginal disc, Dpp is preferentially expressed in the ventral eye domain (Cho et al., 2000). In *dpp* mutants the ventral part of early eye disc exhibits similar pattern defects as seen in *L* mutants. This *dpp* phenotype may be an outcome of ectopic induction of *pnr or wg* expression in the ventral domain as observed in *L* mutants (Singh et al., 2005a). In the DP of early eye imaginal disc Dpp, Hedgehog (Hh) and Wg signaling from the peripodial membrane is required to trigger N activation. Similar to limb patterning and
development (Brook and Cohen, 1996; Penton and Hoffmann, 1996; Theisen et al., 1996), during eye imaginal disc development Dpp antagonizes Wg. This antagonistic interaction occurs in the peripodial membrane across the DV border (Cho et al., 2000). Thus, Dpp signaling plays a role in inducing DV polarity from peripodial membrane.

Ser is the N ligand in the ventral domain of the eye imaginal disc (Table 1.1; Speicher et al., 1994; Cho and Choi, 1998; Dominguez and de Celis, 1998; Papayannopoulos et al., 1998; Dominguez and Casares, 2005). Ser acts downstream of L in the ventral eye as Ser transcription is repressed in early eye discs from L" homozygous larvae. Evidence for this conclusion comes from the Ser-lacZ reporter expression. Interestingly, Ser-lacZ expression in the posterior medial region remains at significant level. Loss-of-function clones of L cause strong reduction of Ser in the ventral eye whereas increased levels of L using the “flp out” approach induce Ser expression even in the dorsal domain of eye imaginal disc. Thus, L that acts downstream to N may be involved in inducing the Ser expression in the developing eye imaginal disc (Chern and Choi, 2002). Hypomorphic alleles of Ser exhibit reduced eye size suggesting a role for Ser in eye development. However, Ser loss-of-function clones do not exhibit any phenotypes in the eye (Sun and Artavanis-Tsakonas, 1996; Papayannopoulos et al., 1998; Chern and Choi, 2002) but misexpression of dominant negative form of Ser (Ser\textsuperscript{DN}) causes severe growth defects in eye imaginal disc (Kumar and Moses, 2001; Singh and Choi, 2003). It is possible that Ser function may be compensated by another factor or Ser may somehow be secreted or transendocytosed into neighboring cells as shown in experiments performed using cell culture system (Klueg and Muskavitch, 1999; Kumar and Moses, 2001; Singh et al., 2005b). This may explain the apparent lack of phenotype in Ser mutant clones. Misexpression of Ser\textsuperscript{DN} in early eye imaginal disc using ey-Gal4 (Hazelett et al., 1998) results in either preferential loss of ventral eye or loss of the entire eye (Singh and Choi, 2003). Misexpression of Ser\textsuperscript{DN} in random gain-of-function clones generated by “flp-out” method (Pignoni and Zipursky, 1997) result in suppression of eye fate in the ventral
half of eye. The similar phenotypes of Ser\textsuperscript{DN} misexpression and L mutants in the eye disc further validate that L and Ser work in the same pathway to regulate the growth of ventral eye domain.

It is known that Ser can activate N only at the DV border since Ser-N interaction is prevented by Fringe (Fng) in the ventral domain cells away from the DV border of the eye imaginal disc. Fng is a glucosaminyltronsferase that elongates O-linked fucose residues to the EGF domains of N (Okajima and Irvine, 2002). Fng is known to bind N to promote N-Delta (Dl) interaction and is required to restrict N activation at the DV border (Irvine and Wieschaus, 1994; Kim et al., 1995; Fleming et al., 1997). Contrary to the positive function of Fng in N-Dl interaction, Fng inhibits Ser-N interaction when it is bound to N protein (Ju et al., 2000; Singh et al., 2005b). As a result the N activation by Dl is enhanced only at the DV border. The expression pattern of these DV patterning genes changes dynamically in the developing eye imaginal disc thereby showing striking differences before and after the initiation of retinal differentiation. Initially, fng is expressed in the ventral domain of eye imaginal disc, but as the eye imaginal disc undergoes retinal differentiation and the morphogenetic furrow proceeds anteriorly, fng expression further evolves. At this stage fng exhibits preferential localization anterior to the furrow in both dorsal and ventral eye domain. These results validate the conclusion of genetic mosaic studies which suggested that DV pattern is established during early eye development prior to retinal differentiation. The essential role of Fng in DV patterning was demonstrated by analysis of fng mutant clones. In loss-of-function clones of fng in the ventral eye, DV polarity is reorganized near the ectopic fng\textsuperscript{+/−} border resulting in non-autonomous polarity reversals. This leads to the generation of de novo equators and ectopic localized activation of N at the fng\textsuperscript{+/−} boundary (de Celis et al., 1996; Cho and Choi, 1998; Go et al., 1998; Baonza and Garcia-Bellido, 2000).
The DV axis in the wing imaginal disc is inverted in comparison to the eye imaginal disc. In the eye imaginal disc, Dl and Ser are preferentially expressed in the dorsal and ventral domains respectively. However, the localization of Dl and Ser preferential expression is reversed in the developing wing imaginal disc. The inversion of the DV axis in the eye and the wing disc may be due to the fact that the eye disc rotates 180° during embryogenesis (Struhl, 1981). Therefore, Ser functions as an N ligand in the dorsal cells whereas Dl is the N ligand in the ventral cells. Not surprisingly, fng is ventral-specific in the eye but dorsal-specific in the wing imaginal disc.

Other candidate genes involved in ventral eye development are Chip and *sloppy paired* (*slp*) (Table 1.1). Chip, a transcriptional co factor is required for the ventral eye development (Roignant et al., 2010). Chip, a ubiquitous transcriptional co-factor, interacts with classes of transcription factor during neural development. Chip has been reported to establish the ventral boundary of the eye and the head tissue (Roignant et al., 2010). Slp belongs to forkhead family of transcription factors which are required for embryonic patterning (Grossniklaus et al., 1992). Slp locus has two transcription units. Both of them are expressed in the ventral eye and are functionally redundant (Sato and Tomlinson, 2007). Slp and Iro-C proteins have been shown to repress each other at the DV midline. N signaling at the DV midline suppress Slp at the midline (Sato and Tomlinson, 2007).

5.2 Dorsal selector genes:

It is known that compartment boundaries are defined by the spatio-temporal expression of genes (Blair, 2001; Curtiss et al., 2002; Dahmann et al., 2011). For example, *engrailed* (*en*) and *apterous* (*ap*) are expressed in the posterior and the dorsal compartments of wing imaginal disc, respectively (Brower, 1986; Cohen et al., 1992; Hidalgo, 1998; Held, 2002). Thus, “Selector” genes were identified that assign a unique property to the cells within their expression domains, which results in the formation of unique territory (Blair, 2001; Curtiss et al., 2002). Moreover,
loss-of-function of these selector genes results in the loss of that particular fate. In the
Drosophila eye, the presence of these selector genes (which exhibit dorsal or ventral domain
specific expression) became apparent in the earlier enhancer trap screens (Bier et al., 1989;
Bhojwani et al., 1995; Sun et al., 1995). Enhancer trap lines containing mini-white (w) and lacZ
reporter gene (P-lacW) (Bellen et al., 1989; Bier et al., 1989; Wilson et al., 1989; Bhojwani et al.,
1995; Sun et al., 1995) that show domain specific expression in the eye were isolated in the
screens. Interestingly, some of these lines have w+ expression restricted only to the dorsal half of
the adult eye. These enhancer trap lines have made significant contributions towards
understanding the DV patterning in the eye (Choi et al., 1996; Sun and Artavanis-Tsakonas,
1996; McNeill et al., 1997; Kehl et al., 1998; Morrison and Halder, 2010). Most of these dorsal
specific P insertion lines were mapped to the chromosomal region 69CD, identifying this region
as a hot spot for P-lacW insertions that show dorsal eye specific expression. The molecular
characterization of this 69CD chromosomal region revealed the existence of a cluster of
homeobox genes, araucan (ara), caupolican (caup) and mirror (mirr) (Gomez-Skarmeta and
Modolell, 1996; McNeill et al., 1997; Grillenzoni et al., 1998; Heberlein et al., 1998; Kehl et al.,
1998; Singh et al., 2005b). This cluster of homeobox gene is located within an approximately
140Kb region (Netter et al., 1998), are expressed in the dorsal half of the eye. They are referred to
as Iroquois –complex (Iro-C) as the mutation in these genes lack lateral thoracic bristles and
resemble the hair style of the Indian tribe, the Iroquois, also called as Mohawks (a native tribe
which shaved all but a medial stripe of hairs on the head) (Gomez-Skarmeta and Modolell, 1996;
Leyns et al., 1996). They named the genes Araucan and Caupolican in honor of Amerindian
tribes: Aracaunians and one of their heroes- Caupolican.

The members of Iro- complex are highly conserved essential genes and exhibit significant
differences in their expression pattern (Gomez-Skarmeta and Modolell, 2002). Mirr is strongly
and dynamically expressed in the CNS (Netter et al., 1998; Urbach and Technau, 2003) and is
essential for follicle cell patterning (Jordan et al., 2000) while Ara and Caup are preferentially expressed in mesodermal tissues in the embryos (Netter et al., 1998). However, in the eye imaginal disc all three Iro-C members are expressed in the dorsal half (Figure 1.6E), raising a possibility that they might be functionally redundant. Loss-of-function of the mirr<sup>e48</sup> allele shows weak but significant defects of non-autonomous DV polarity reversals in comparison to mirr<sup>+</sup> ommatidia in the dorsal half of the eye (McNeill et al., 1997). Compartments of different cell lineages do not intermingle due to differences in cell identities and affinities (Garcia-Bellido et al., 1973; Irvine, 1999; Dahmann et al., 2011). Somatic clones of cells lacking mirr function in dorsal half of eye exhibit smooth clone borders, indicating that cells lacking mirr avoid mixing with the neighboring mirr expressing cells. However, the clones in the ventral half where mirr is not expressed show wiggly clone borders (Yang et al., 1999). This analysis suggests that mirr functions as a dorsal fate selector. Since the phenotype of mirr clones was not strong enough it raised the possibility that ara and caup, the other two members of Iro-C, can partly compensate for the loss of mirr function in the eye. The issue of functional redundancy got resolved when a deficiency iro<sup>DMF3</sup>, which uncovers all three Iro-C genes by the deletion of ara and caup as well as a 5’-region of mirr (Gomez-Skarmata et al., 1996; Diez del Corral et al., 1999), was employed for clonal analysis. Loss-of-function clones of iro<sup>DMF3</sup> in the eye showed repolarization of the ommatidial polarity in the dorsal clones along with dorsal eye enlargement or formation of an ectopic eye field on the dorsal margin. There was no phenotype in the ventral half of the eye (Figure 1.6F, G). These results further highlighted the importance of boundary between the dorsal and ventral cell types. These results strongly support that the three Iro-C genes are partially redundant and the Iro-C as a whole is required for organizing the DV polarity pattern and growth of the eye.

Loss-of-function of iro<sup>DMF3</sup> also suggested that Iro-C genes function as dorsal selectors for head structures as well since mutant clones in the dorsal region induces the formation of ventral
head structures (Cavodeassi et al., 2000). Ectopic ventral head tissues resulted from loss of *Iro-C* genes are cell-autonomous and therefore accompanied by loss of corresponding dorsal structures. In contrast, ectopic ventral eyes are generated non-cell autonomously since reversals of DV ommatidial polarity are detected in *Iro-C*+ wild-type region adjacent to the mutant clones. This also supports the idea that the DV boundary is an organizing center for DV pattern and growth in the eye imaginal disc. Furthermore, DV patterning of the eye occurs in earlier larval stages than the head patterning. In the *Drosophila* eye, *pnr* another dorsal gene, which is expressed in the dorsal eye margin (Figure 1.6A), exhibits similar loss-of-function (Table 1.1; Figure 1.6B, C) and gain-of-function (Figure 1.6D) phenotypes as observed with *Iro-C* in the eye and the head (Maurel-Zaffran and Treisman, 2000; Pichaud and Casares, 2000; Singh et al., 2005b; Oros et al., 2010). *Pnr*, a GATA-1 transcription factor, plays an important role in the dorsal eye development, and acts as a selector for the dorsal eye fate (Ramain et al., 1993; Maurel-Zaffran and Treisman, 2000; Pichaud and Casares, 2000; Dominguez and Casares, 2005; Singh et al., 2005b; Oros et al., 2010). In the hierarchy of dorsal genes, *pnr* is the top most gene, and induces Wingless (Wg) which in turn induce the expression of downstream target genes *mirr* in the dorsal half of the eye (Maurel-Zaffran and Treisman, 2000; Dominguez and Casares, 2005; Singh et al., 2005b). During later stages of development which corresponds to the retinal differentiation stage in late second instar and third instar of larval eye development, *pnr* is involved in defining the dorsal eye margin by regulating the retinal determination (RD) genes (Oros et al., 2010).

Wg, a secretory protein and a morphogen, is expressed along the antero-lateral margins of the third instar eye imaginal disc (Table 1.1; Baker, 1988). Wg plays multiple roles during eye development. One of these roles of Wg is to promote growth of early eye imaginal disc. During early eye development Wg expression is restricted to the dorsal eye domain (Cho et al., 2000; Chang et al., 2001). During retinal differentiation stage, Wg is known to prevent ectopic induction of retinal differentiation from the lateral eye imaginal disc margin (Ma and Moses,
1995; Treisman and Rubin, 1995). Thus, Wg that acts as a negative regulator of eye during retinal differentiation functions as a dorsal eye fate gene. Dl, an N ligand in the dorsal eye imaginal disc, has been assigned to the dorsal gene category in the early eye imaginal disc (Table 1.1). Dl is preferentially expressed in dorsal domain of eye imaginal discs during first and second instar stages.

6. Genes with domain specific growth response: Role of teashirt (tsh) and homothorax (hth)

In addition to the genes that exhibit DV domain specific expression during patterning, there is a group of genes, which show differential functions in the dorsal-ventral compartments but are not expressed in a DV specific pattern. These genes can be broadly classified into two groups: (I) Genes expressed uniformly in the eye imaginal disc but their functional domain is restricted only to the ventral half of the eye, for example L (described in Section 4, 5.1) and hth (Table 1.1). (II) Genes that are expressed uniformly in the early eye imaginal disc and functions differently in the dorsal and ventral half of the eye, for example, tsh (Table 1).

I. Hth: Hth a vertebrate homolog of murine proto-oncogene MEIS1 (Moskow et al., 1995), encodes a homeodomain transcription factor of the three-amino-acid extension loop (TALE) subfamily (Rieckhof et al., 1997). Like L, hth is expressed in the entire early eye primordium. However, with the onset of differentiation in the eye Hth expression gets restricted to the cells anterior to the furrow (Pai et al., 1998; Pichaud and Casares, 2000; Bessa et al., 2002; Singh et al., 2002a). Even though hth is expressed anterior to the furrow both in the dorsal and ventral half of the eye imaginal disc, the loss-of-function of hth causes eye enlargement only in the ventral eye margin (Figure 1.7B, B’). However, loss-of-function clones of hth in the dorsal compartment do not show any phenotype in the eye imaginal disc. Furthermore, hth mutant cells do not survive in the anterior eye (Pichaud and Casares, 2000; Bessa et al., 2002; Bessa et al.,
2008). As expected misexpression of hth suppresses the eye fate (Pai et al., 1998). Moreover, eye suppression function of Hth is independent of any domain constraint. Hth is involved in multiple functions during development and is required for nuclear localization of a homeoprotein Extradenticle (Exd). Hth encodes a protein with nuclear localization signal (NLS) and two conserved domains: the N terminal evolutionarily conserved MH domain (for Meis and Hth), and a C-terminal region including the homeodomain (HD) (Rieckhof et al., 1997; Kurant et al., 1998; Pai et al., 1998; Noro et al., 2006). Alternative splicing is known to provide additional complexity to the genes encoding the Hth transcription factors (Glazov et al., 2005; Noro et al., 2006). Hth forms a heterodimer with Exd through its MH domain and translocates into the nucleus to regulate transcription (Ryoo et al., 1999; Jaw et al., 2000; Stevens and Mann, 2007). Since Exd is present in entire eye, the ventral specific function of hth has been proposed through its interaction with Wg and Tsh. Together they are involved in suppression of eye fate on the ventral margin.

II. Tsh: The homeotic gene tsh encodes a C2H2 zinc-finger transcription factor with three widely spaced Zinc finger domains (Fasano et al., 1991). Tsh plays an important role during Drosophila eye development (Pan and Rubin, 1998; Bessa et al., 2002; Singh et al., 2002a; Singh et al., 2005b; Datta et al., 2009; Kumar, 2009; Kumar, 2010; Kumar, 2011). tsh is expressed both in dorsal and ventral eye anterior to the furrow and exhibits a DV constraint in its function. In the ventral eye, tsh acts as repressor of eye fate whereas in the dorsal eye it promotes eye development (Singh et al., 2002b; Singh et al., 2004). Interestingly, the DV constraint in tsh function in the eye stems from the partners with which it collaborate in the dorsal or the ventral eye disc (Figure 1.8; Singh et al., 2004). It was shown that Tsh cooperates with Iro-Complex members and Dl in the dorsal eye for its growth promotion function (Singh et al., 2004). The function of tsh in the ventral eye is dependent on Hth and Ser. The expression of
tsh overlaps with hth in the eye imaginal disc, and like hth, tsh expression also evolves during larval eye development. Initially, in first instar eye imaginal disc tsh is expressed in the entire eye imaginal disc but its expression retracts anteriorly to nearly three quarters of the eye imaginal disc when the retinal differentiation begins (Bessa et al., 2002; Singh et al., 2002a). Furthermore, Tsh and Hth physically interact with each other [along with Pax-6 homolog, Eyeless (Ey)] to repress the expression of downstream target genes (Bessa et al., 2002; Dominguez and Casares, 2005). Further insights into the potential mechanism of tsh and hth in regulating growth and differentiation in the eye came initially from analysis of expression patterns of the retinal determination (RD) gene network members (Bessa et al., 2002). It has been proposed that Tsh, Hth and Ey coexpress in the proliferating cells anterior to furrow to block precocious retinal differentiation and promote cell proliferation (Bessa et al., 2002; Singh et al., 2002a; Dominguez and Casares, 2005). All these studies suggest that DV patterning genes contribute towards the growth of the eye field.

7. **Boundary formation during organogenesis**

One of the important questions is how organ size and growth regulated by DV patterning genes in the eye. The dorsal selector genes assign a dorsal fate and thereby generate a group of cells with unique properties that makes them different from the default ventral state cells of the developing eye disc. Interestingly, the boundary between the dorsal and ventral cells is maintained by the antagonistic interactions between the genes required for the growth and development of the dorsal and ventral domains of the eye (Figure 1.9; Singh et al., 2005a). It has been shown that L is essential for growth of the ventral eye tissue but is dispensable in the dorsal region specified by pnr function (Singh and Choi 2003). In addition to a boundary between the dorsal and ventral compartment within the eye, a boundary is defined between the developing eye
field and the surrounding head cuticle on the dorsal and ventral margins. Since the adult eye, head cuticle and other mouthparts are generated from the eye-antennal imaginal disc, there is a sequential fate restriction between the developing eye and head cuticle. Interestingly, these DV patterning genes play an important role of defining the boundary of the eye field on the dorsal and the ventral margins (Oros et al., 2010).

The boundary between the eye field and the head cuticle on the dorsal margin is regulated by pnr. It has been shown that pnr function evolves during eye development. During early second instar of development pnr is required for defining the dorsal lineage, before the onset of retinal differentiation, by inducing Wg and members of the Iro-C complex (Maurel-Zaffran and Treisman, 2000; Singh and Choi, 2003; Singh et al., 2005b; Oros et al., 2010). However, later during the late second- instar stage of eye development, when the morphogenetic furrow (MF) is initiated, pnr suppresses the photoreceptor differentiation at the dorsal eye margin (Oros et al., 2010). The endogenous expression of pnr in only in the peripodial membrane of the dorsal eye margin which give rise to the adult head cuticle. Loss-of-function clones of pnr exhibit ectopic dorsal eyes, which are restricted within the clones, suggests that absence of pnr function promotes ectopic eye formation in the dorsal eye margin. Thus, Pnr defines the boundary between the head cuticle and the dorsal margin of the developing eye field (Oros et al., 2010). Since pnr is not expressed in the ventral eye there is a different mechanism to define the boundary of eye field on the ventral margin. The boundary of eye field on the ventral eye margin is defined by the antagonistic interaction of L with hth (Singh et al., 2011). In the ventral eye, transcriptional cofactor Chip interacts with the LIM-homeodomain proteins to define the boundary of the eye field (Roignant and Treisman, 2009). Interestingly, Chip mediated regulation of the ventral eye boundary is independent of hth (Roignant and Treisman, 2009). Thus, the genetic cascade regulating the boundary of eye field on the dorsal and the ventral margin of the eye is different.
8. Concluding Remarks

Our understanding of the axial patterning of the *Drosophila* eye is far from complete. In this review, we have described the overview of key developmental events and genes involved in early DV patterning. The DV compartment formation is a key to initiate patterning and growth in the early eye imaginal disc. The present information clearly illustrates that DV patterning is required to initiate the generation of heterogeneous population (dorsal and ventral cell fate) of cells within a homogenous (default ventral fate) early eye primordial. Although our knowledge on the DV patterning in the eye has dramatically increased in recent years, we still do not know the molecular interactions important for the regulation of DV patterning. Moreover, many more genes (both known and novel) are expected to be involved in DV patterning, and future studies using novel genetic and bioinformatics approaches should help in defining the full complement of genes involved in this intricate process. Identification and functional analysis of more molecular players involved in this process will help provide a better picture of how a small number of cells in the disc primordium grow to form a precise pattern of mirror symmetry in the compound eye. Furthermore the possibility of crosstalk of the DV patterning pathway with other signaling pathways to regulate growth during early phase of eye development cannot be ruled out. All these information will lay foundation about understanding the process of organogenesis, as loss-of-function of the genes involved in DV patterning results in the loss of the eye field or a part of the eye field. The complexity and precision of the neural connectivity in the adult visual system has fascinated researchers for a long time. The DV polarity of the retina is responsible for controlling the targeting of the retinal axon projections to the brain in humans and other higher vertebrates. Thus, DV patterning genes also contribute towards the wiring of the brain to the retina. How all these different facets work together to define the final form of this complex structure eye is an open question and is of fundamental importance.
8.1 Similarities with vertebrate eye

The basic sensory epithelium design of the vertebrate and most invertebrate eyes including *Drosophila* eye is similar (Charlton-Perkins and Cook, 2010; Sanes and Zipursky, 2010). The morphogenetic furrow (MF) in the fly eye is analogous to the wave of neurogenesis in the vertebrate retina (Neumann and Nüsslein-Volhard, 2000; Hartenstein, 2002). Recent studies in the vertebrate visual systems have identified several genes that are expressed in a DV domain specific manner in the retina. BMP4, a TGF-β closely related to Dpp, has been implicated in development of progenitor cells in the dorsal half of the eye and in establishment of the DV axis of the retina which the eye in *Xenopus* (Papalopulu and Kintner, 1996). The dorsal selectors of the vertebrate eye, BMP-4 and TbX5 acts to restrict the expression of Vax2 and Pax2 to the ventral domain of the eye (Koshiba-Takeuchi et al., 2000; Mui et al., 2002; Peters, 2002; Peters and Cepko, 2002). These DV expression domains correspond to the developmental compartments (Peters, 2002; Peters and Cepko, 2002). The DV patterning plays an important role in retinotectal projection pattern (Koshiba-Takeuchi et al., 2000; McLaughlin et al., 2003). The R-cell projections, form a precise topographic connection with the optic lobe, and are referred to as retinotopy, which is common to both the vertebrate and the insect visual system (Gaul, 2002). Furthermore, Jagged-1(Jag1), a vertebrate homolog of the *Drosophila* ventral eye gene Ser, shows a DV asymmetric expression pattern in the retina. Moreover, the loss-of-function of Jag1 results in Alagille’s syndrome, which also affects the eye (Oda et al., 1997; Xue et al., 1999; Kim and Fulton, 2007). Interestingly, it has been shown that mouse retina also begins with a default ventral like state (Murali et al., 2005). Therefore, the DV boundary may play conserved roles in organizing growth and pattern of visual system in higher animals, and studies in *Drosophila* will further our knowledge in the area of animal development mechanisms and help to unravel the genetic underpinnings of developmental defects caused by mutations in human homologs of *Drosophila* DV patterning genes.
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Fig.1.3. *Drosophila* eye is a highly organized structure. (A-C) Eye antennal imaginal disc of a third instar larva. (B) Eye imaginal disc stained for membrane marker Disc large (Dlg: green) and Elav (red), a pan-neuronal marker that marks the photoreceptor neurons are shown. Arrowhead marks the position of the morphogenetic furrow (MF). (C, C’) Eye imaginal disc stained with Bar (B: green) to mark DV polarity. (D) Scanning electron micrograph (SEM) of the wild-type *Drosophila* eye. Adult eye is a compound eye which is made up of 750-800 unit eyes, each termed as an ommatidium (Wolff and Ready, 1993). All ommatidia are organized as a regularly spaced hexagon arranged in mirror image symmetry along the dorso-ventral (DV) axis. (E) Cross section of the adult compound eye showing arrangement of ommatidial clusters along DV axis (Wolff and Ready, 1993). Note that in each ommatidium, eight rhabdomeres are organized in an asymmetric trapezoidal fashion. All these ommatidia within a compound eye are organized in two basic clusters based on the orientation of the trapezoid. If the R3 rhabdomere points up it is dorsal whereas the reverse is ventral. (F) Cartoon showing the mirror image symmetry of ommatidial cluster orientation in the dorsal and ventral half of the adult eye. All the blue arrows mark the ommatidia in the dorsal half of the eye, while the red ones mark the ventral half of the eye. All images are oriented as Dorsal (up), Ventral (down), Anterior (right), and Posterior (left). [AN: antenna]
Fig.1.4. Ventral is the default state of the early eye imaginal disc. Larval eye primordium comprising of a few cells require the function of ventral genes L/Ser for growth and proliferation (Singh and Choi, 2003). Loss-of-function phenotype of L/Ser in the developing eye imaginal disc evolves along the temporal scale. During early first instar of larval development, loss-of-function of L/Ser results in complete loss of the eye field. However, after the onset of pnr expression during early second larval instar, which results in generation of DV lineage in the developing eye imaginal disc, loss of L/Ser results in loss of only the ventral half of the eye. However, in late third instar stage of development when the retinal differentiation is almost complete loss of L/Ser does not have significant affect on the overall adult eye morphology. Based on these results it was proposed that the entire early eye primordium, prior to onset of pnr expression, is ventral in fate (Singh and Choi, 2003).
Fig.1.5. **Lobe (L) and Serrate (Ser)** are required for cell survival in developing eye imaginal disc. (A) In wild-type eye imaginal disc, L (green) expression is ubiquitous. Elav (red) marks the photoreceptor neurons. (B) wild-type adult eye. (C, D) Loss of L results in the preferential loss of ventral half of the (C) developing eye imaginal disc, and (D) the adult eye. (C) Eye imaginal discs stained for Wg (green) to identify dorsal versus ventral eye imaginal disc compartment. The boundary of the eye field is as outlined in C (white) and D (black) showing preferential loss of ventral eye. (E, F) Early loss-of-function of Ser by misexpressing dominant negative form of Ser in the entire eye imaginal (Kumar and Moses, 2001; Singh and Choi, 2003) using an *ey*-Gal4 driver (Hazelett et al., 1998; Singh and Choi, 2003) results in complete loss of eye field both in (E) the eye imaginal disc, and (E) the adult.
Fig.1.6. Pnr and Iro-C members function as dorsal eye fate selectors. (A) Pnr expression (green) is restricted to the dorsal eye margin of developing eye imaginal disc (Maurel-Zaffran and Treisman, 2000; Pichaud and Casares, 2000; Singh and Choi, 2003). Elav (red) marks the photoreceptor neurons. (B, C) Loss-of-function clones of pnr results in the enlargement of existing dorsal eye field (e.g., in the clone outlined in B) in eye imaginal disc (B) and adult eye(C). (B) Note that there is a non-autonomous eye enlargement in eye imaginal disc, which is attributed to generation of de novo equator in the dorsal compartment of eye imaginal disc (Maurel-Zaffran and Treisman, 2000; Oros et al., 2010). (D) Misexpression of pnr (ey>pnrD4) in the eye imaginal disc suppresses the eye fate validating a late function of pnr in defining the eye field boundary (Oros et al., 2010). (E) The expression domain of the members of Iroquois complex (Iro-C>GFP, green) spans the dorsal region of the eye imaginal (G, H) Loss-of-function of Iro-C causes dorsal eye enlargements in the (G) eye imaginal disc and in (H) adult eye. These phenotypes are similar to the (B, C) pnr loss-of-function phenotypes. (H) Misexpression of ara, a member of Iro-C, in entire eye imaginal disc (ey>ara) results in small eye. [D: Dorsal, V: Ventral]
Fig.1.7. **Domain specific function of hth is restricted to the ventral eye margin.** (A, A’) Hth (green) is expressed anterior to the furrow both in the dorsal as well as ventral domain of the eye imaginal disc (Rieckhof et al., 1997; Pai et al., 1998; Pichaud and Casares, 2000; Bessa et al., 2002; Singh et al., 2002a). ELAV (red), a pan neural marker, marks the photoreceptors neurons in the eye imaginal disc. (A’) Note that Hth is expressed in the peripodial membrane (PM). (B, B’) Loss-of-function clones of hth marked by the absence of the GFP reporter (clonal boundary marked by white dotted line) in the ventral eye results in eye enlargement whereas in the dorsal eye these clones do not have any effect. (C, D) Misexpression of hth in the entire eye using ey-Gal4 driver (ey\textgreater hth) results in reduced eye field as seen in (C) eye imaginal disc and (D) adult eye (Pai et al., 1998).
Fig.1.8. DV asymmetric function of homeotic gene teashirt (tsh) in the developing eye imaginal disc depends on the spatial cues provided by other genes which are expressed in DV asymmetric fashion (Singh et al., 2004). Gain-of-function of Tsh suppresses the eye fate in the ventral eye whereas it promotes dorsal eye enlargement. It has been shown the DV asymmetric function of Tsh in the developing eye imaginal disc depends on the domain specific cues provided by genes that are either expressed or function in a DV asymmetric fashion. Tsh in collaboration with Wg and Ser can induce Hth in the ventral eye to suppress the eye fate. The ventral eye suppression function of tsh is independent of other ventral specific genes like fng and L. In the dorsal eye, tsh is known to promote growth and eye in collaboration with Iro-C members and a N ligand in the dorsal eye, Dl (Singh et al., 2002; Singh et al., 2004; Singh et al., 2005b). [D: Dorsal, V: ventral]
### DV asymmetric functions of *teashirt (tsh)* in *Drosophila* eye

| *teashirt (tsh)* in collaboration with | Dorsal eye selectors \{
<table>
<thead>
<tr>
<th></th>
<th>*aracuan (ara), mirror (mirr), caupolican (caup)} and delta (dl)</th>
<th>Promote dorsal eye outgrowth.</th>
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<tr>
<td></td>
<td><em>homothorax (hth)</em> wingless (wg) Serrate (Ser)</td>
<td>Suppress ventral eye development</td>
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Fig.1.9. Genes with DV asymmetric expression or function are involved in regulating DV patterning in the Drosophila eye. (I) Early eye imaginal disc primordium comprises of a homogeneous population of ventral eye fate cells whose growth and survival depends on L/Ser function (Singh and Choi, 2003; Singh et al., 2005b; Singh et al., 2006). (II) Later during development, DV lineage is generated by onset of expression of dorsal gene pnr (Singh and Choi, 2003). Pnr acts upstream of Wg, which in turn triggers the expression of downstream Iro-C members (Maurel-Zaffran and Treisman, 2000). Dl, an N ligand, is involved in dorsal eye development. The default ventral eye fate is maintained by function of L and Ser (Singh and Choi, 2003; Singh et al., 2005b). There are three other players involved in ventral eye development viz., fringe (fng) (Cho and Choi, 1998; Dominguez and de Celis, 1998; Papayannopoulos et al., 1998; Irvine, 1999; Cho et al., 2000; Dominguez and Casares, 2005), Chip (Roignant et al., 2010) and Sloppy paired (Slp) (Sato and Tomlinson, 2007). L is known to act antagonistically to the dorsal genes to define the boundary between the dorsal and ventral compartment of the eye (Singh et al., 2005a). L and Ser also interact antagonistically with hth on the ventral eye margin to define the ventral eye margin boundary (Singh et al., 2005b). There is a positive feedback loop between Wg and Hth on the ventral eye margin (Pichaud and Casares, 2000; Singh et al., 2004; Dominguez and Casares, 2005; Singh et al., 2005b). Chip act independently of hth (Roignant et al., 2010). Sloppy paired (Slp) is involved in ventral eye development by repressing Iro-C at the DV midline (Sato and Tomlinson, 2007). These DV patterning genes work together and contribute towards sculpting the final shape and size of the adult eye. (III) The ommatidial clusters within an adult eye are organized into two mirror symmetric orientations that are polarized in the opposite directions in the dorsal and the ventral half. The dorsal selectors exhibit a dorsal specific expression of mini-white reporter gene in the adult eye.
Table 1.1. Genes involved in dorso-ventral patterning and domain specific expression and growth.

<table>
<thead>
<tr>
<th>Drosophila Homolog</th>
<th>Vertebrate Homolog</th>
<th>Nature</th>
<th>Function in eye</th>
<th>References</th>
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<tr>
<td><strong>Ventral Genes</strong></td>
<td></td>
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<tr>
<td>fringe (fng)</td>
<td>Lunatic fringe</td>
<td>Glycosyl transferase</td>
<td>Secreted signaling protein, DV boundary formation</td>
<td>(Irvine and Wieschaus, 1994; Cho and Choi, 1998; Dominguez and de Celis, 1998; Papayannopoulos et al., 1998)</td>
</tr>
<tr>
<td>Serrate (Ser)</td>
<td>Jagged-1</td>
<td>Ventral N ligand</td>
<td>Ventral eye growth and development</td>
<td>(Speicher et al., 1994; Cho and Choi, 1998; Dominguez and de Celis, 1998; Papayannopoulos et al., 1998; Cho et al., 2000)</td>
</tr>
<tr>
<td>Chip</td>
<td>Nli/Ldb1/Clim-2</td>
<td>Transcription co-factor</td>
<td>Define ventral eye boundary</td>
<td>(Roignant et al., 2010)</td>
</tr>
<tr>
<td>Sloppy paired (Slp2)</td>
<td>BF-1 (not complete homology)</td>
<td>Forkhead transcription factor</td>
<td>Ventral eye growth</td>
<td>(Sato and Tomlinson, 2007)</td>
</tr>
<tr>
<td>decapentaplegic (dpp)</td>
<td>BMP</td>
<td>TGF-β</td>
<td>Ventral growth</td>
<td>(Chanut and Heberlein, 1997; Singh et al., 2005b)</td>
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<tr>
<td><strong>Dorsal Genes</strong></td>
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<tr>
<td>pannier (pnr)</td>
<td>GATA-4</td>
<td>Zinc finger, GATA family</td>
<td>Dorsal eye fate selector</td>
<td>(Ramain et al., 1993; Maurel-Zaffran and Treisman, 2000; Gomez-Skarmeta and Modolell, 2002; Singh et al., 2005b; Oros et al., 2010)</td>
</tr>
<tr>
<td>Drosophila</td>
<td>Vertebrate Homolog</td>
<td>Nature</td>
<td>Function in eye</td>
<td>References</td>
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<tr>
<td>Caupolican (caup)</td>
<td>Irx2, 5</td>
<td>homeodomain</td>
<td>Dorsal eye fate selector</td>
<td>(Gomez-Skarmeta and Modolell, 1996; Cavodeassi et al., 1999; Pichaud and Casares, 2000; Gomez-Skarmeta and Modolell, 2002)</td>
</tr>
<tr>
<td>mirror (mirr)</td>
<td>Irx 4, 6</td>
<td>homeodomain</td>
<td>Dorsal eye fate selector</td>
<td>(McNeill et al., 1997; Heberlein et al., 1998; Kehl et al., 1998; Yang et al., 1999; Singh et al., 2005b)</td>
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<tr>
<td>Genes with DV asymmetric response</td>
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<tr>
<td>homothorax (hth)</td>
<td>Meis</td>
<td>homeodomain</td>
<td>Ventral eye suppression</td>
<td>(Rieckhof et al., 1997; Pai et al., 1998; Ryoo et al., 1999; Pichaud and Casares, 2000; Dominguez and Casares, 2005; Singh et al., 2005b; Bessa et al., 2008)</td>
</tr>
<tr>
<td>teashirt (tsh)</td>
<td>TSH1, TSH2, TSH3</td>
<td>C2H2 zinc finger transcription factor</td>
<td>Dorsal eye growth, ventral eye suppression</td>
<td>(Fasano et al., 1991; Pan and Rubin, 1998; Bessa et al., 2002; Singh et al., 2002; Singh et al., 2004; Datta et al., 2009)</td>
</tr>
<tr>
<td>Lobe (L)</td>
<td>PRAS40 (Ortholog)</td>
<td>Proline rich Akt substrate</td>
<td>Ventral eye growth, no affect on dorsal eye</td>
<td>(Chern and Choi, 2002; Singh and Choi, 2003; Singh et al., 2005a; Singh et al., 2005b; Singh et al., 2006; Wang and Huang, 2009)</td>
</tr>
<tr>
<td>Expression on both margins</td>
<td>optomotor blind (omb)</td>
<td>Tbx5</td>
<td>Transcription factor</td>
<td>Expressed on dorsal and ventral eye margin. Not known.</td>
</tr>
</tbody>
</table>
C. Research Objectives

The mechanisms underlying cellular processes during organogenesis are conserved across the species. Genetically controlled events including axial patterning, growth and differentiation are required for the formation of organ primordium. Out of these events, axial patterning is required for transition of single sheet of epithelial cells into a three dimensional organ. Axial patterning is the lineage restriction event and results in generation of dorsal-ventral, anterior-posterior and proximal-distal axes of the organ. Patterning is strictly regulated by several genes during different stages of the development. Several signaling pathways are regulated in domain specific manner which are required for survival function and preventing cell death in the respective domains at different developmental time windows. Any abnormalities in process of axial patterning may lead to complete loss or malfunctioning of the organs. However, the complete array of genes involved and their interactions in order to define the different axes are not completely understood yet.

In the present work, we describe the role of an E3 ubiquitin ligase, Cullin-4 (Cul-4) in preventing cell death by regulating asymmetric expression of Wingless and thereby promoting cell survival in the ventral domain of the developing eye. We provide multiple evidences to assign the role to Cul-4 in promoting ventral eye development in Chapter 3 (Manuscript in preparation).

We describe the role of GATA-1 transcription factor *Pannier* (*pnr*) for downregulating the retinal determination pathway via suppressing zinc finger transcription factor *teashirt* (*tsh*) during dorsal eye development in *Drosophila* (Oros and Tare et al., 2010). Thus in Chapter 4, we describe role of *pnr* in suppression of the dorsal eye field.

In Chapters 3 and 4, we have used the *Drosophila* eye as a model system, to understand the early event of axial patterning that occurs prior to differentiation of the
photoreceptors. In Chapter 5, we describe a relatively late condition of neurodegeneration in the differentiated photoreceptor neurons of the eye. We show that under condition of Amyloid Beta 42 (Aβ-42 peptide) toxicity, neuronal cell death occurs via induction of JNK signaling pathway in case of Alzheimer’s Disease (AD). These studies might be beneficial in future to find regulatory molecules of JNK signaling as therapeutic agents in case of Alzheimer’s.
CHAPTER 2
MATERIALS AND METHODS

2.1 Fly Stocks
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2.3 Twin Spot Analysis
2.4 Immunohistochemistry
2.5 TUNEL Assay for detection of Cell Death
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2.1 Fly stocks

\[ y^1; P\{SUPor-P\}Cul-4^{KG2900}/CyO; ry^{506} \] (Bellen et al., 2004), \[ cul-4^{11L}/CyO-GFP \] (Hu et al., 2008), \[ cul-4^{ExG1}/CyO \], \[ cul-4^{HJ11}/twi-GFP \], \[ CyO, cul-4^{ExL2}/CyO \], \[ cul-4^{ExG3}/CyO \] (Lin et al., 2009), \[ y, w; FRT82B pnr^{vx6}/CyO \] (Heitzler et al., 1996), \[ y, w, eyFLP; FRT 42D cl-w^+/CyO \] (Newsome et al., 2000), \[ EP 2518 \{ (UAS-cul-4 (Rorth, 1996)) \}, \[ L2/CyO; ey GAL4 \] (Singh et al., 2005), \[ wg-LacZ/CyO \] (Kassis et al., 1992), \[ UAS-sgg^A9 \] (Hazelett et al., 1998), \[ UAS-arm \] (Zecca et al., 1996), \[ UAS-dTCR^{DN} \] (van de Wetering et al., 1997), \[ UAS-P35 \] (Hay et al., 1994), \[ Df(3L)H99/TM6B \] (Abbott and Lengyel, 1991), \[ puc^{Egg} \] (Martin-Blanco et al., 1998), \[ UAS-puc \] (Martin-Blanco et al., 1998), \[ UAS-bsl^{DN} \] (Adachi-Yamada et al., 1999), \[ UAS-DJun^{aspv7} \] (Treier et al., 1995), \[ UAS-pnr^{D4} \] (Haenlin et al., 1997), \[ UAS-ara \] (Gomez-Skarmeta and Modolell, 1996), \[ UAS-hth^{12} \] (Pai et al., 1998), \[ UAS-hth^{ENR} \] (Gomez-Skarmeta and Modolell, 1996; Inbal et al., 2001), \[ y, w; tsh^8/CyO \] (Fasano et al., 1991), \[ UAS-tsh \] (Gallet et al., 1998), \[ UAS-ds tsh \] Casares (Bessa and Casares, 2005), \[ y, w; tsh^{AS} \] (Sun et al., 1995), \[ UAS-NLS-GFP^{S65T} \] (Ito et al., 1997), \[ UAS-wg \] (Azpiazu and Morata, 1998), \[ pnr-Gal4, UAS-GFP \] (Singh et al., 2005), \[ UAS-pnr^{ENR} \] (Klinedinst and Bodmer, 2003), \[ UAS- Aß42 \] (Casas-Tinto et al., 2011; Cao et al., 2008)

2.2 Mosaic Analysis

In order to generate loss-of-function clones, we have used genetic mosaic techniques available to study gene function during *Drosophila* development. The Flp/FRT system in *Drosophila* allows abolishing function of an embryonic lethal gene. In this system, Flippase \((\text{Flp})\) enzyme catalyzes mitotic recombination between homologous chromosomes harboring Flp Recombinase Targets \((\text{FRTs})\) that are inserted near centromeres. The portion of the chromosome arm distal to the mitotic recombination site becomes homozygous mutant for the gene of interest because the FRT sites are always located close to the centromere on chromosome arms. The Flippase enzyme in the system can be placed under a tissue-specific promoter such as *eyeless*.
(ey) (or any other promoter) for targeted knock down of specific genes in the eye (Newsome et al., 2000). A mini-white transgene is usually placed onto these arms so that the mutant (−/−) and wild-type (+/+) twin-spot clones can be identified in the heterozygous background (+/−). Thus, a clone of mutant cells in the eye appears unpigmented (white), whereas the wild-type twin-spot clone would be a darker shade of red than the surrounding heterozygous tissue. For our studies, we have used transgenes which also harbor GFP as reporter such that in the eye imaginal discs, homozygous clones of mutant cells (−/−) populations lack GFP completely versus the wild-type (+/+) twin spot populations which are GFP positive or heterozygous (+/−) for the mutant gene {Xu and Rubin, 1993; Xu and Rubin, 2012; Golic, 1991}, (Fig. 2.1).

We have used a modification of this basic Flp-FRT system in order to study loss-of-function of cul-4. We were not able to recover mutant populations of the cells in the eye and hence we used cell lethal white plus technique (cl-w+) where only mutant cells for the specific genes survive and wild-type cells are otherwise killed by cell-lethal approach (Newsome et al., 2000).

To generate loss of function clones of cul-4 in the eye, virgins of eyFlp; FRT42D, Cl-w+/CyO-GFP were crossed to i) FRT 42D, cul-4 ExG1-3/CyO, ii) FRT 42D, cul-4 ExG3-5, iii) FRT 42D, cul-4 ExL2-1/CyO and, iv) FRT 42D, cul-4 j111/twi>GFP, CyO.

To generate loss of function clones of pnr in the eye, virgins flies of eyFlp; FRT82B, ubi GFP were crossed to males of y, w; FRT82B pnr^{yn5}/CyO, (Heitzler et al., 1996).

**Targeted Misexpression Studies**

To carry out targeted misexpression studies, we have used Gal4/UAS system for (Brand and Perrimon, 1993). We have used ey-Gal4 (Hazelett et al., 1998), bi-Gal4 (Calleja et al., 1996) in order to misexpress the UAS-transgenes. The GAL4/UAS system exploits the sensitivity of the
UAS enhancer element to the GAL4 transcription factor from yeast. It is used to target the misexpression of the gene of interest. The coding region of a gene (X in Fig.2.2) is linked to UAS and the GAL4 is expressed under the tissue specific enhancer of a gene. Consequently the gene of interest (X) is overexpressed in the targeted domains of the tissue specific enhancer {(Brand and Perrimon, 1993); (Blair, 2003); (Duffy, 2002)}.

For misexpression of Aβ-42 transgene, experiments were conducted using the Glass Multimer Reporter driver line (GMR-Gal4), which directs expression of transgenes in the differentiating retinal precursor cells of the developing eye (Moses and Rubin, 1991). All Gal4/UAS crosses were done at 18°C, 25°C and 29°C, unless specified, to sample different induction levels.

In order to carry out mosaic analysis and targeted misexpression studies together in the developing tissues, we have generated a new tool using bi-Gal4 driver (Calleja et al., 1996). Generation of this tool helped us analyzing function of specific genes in domain specific manner. Details of generation and usage of this tool have been described in details in (Tare et al., 2013) (attached).

**Twin Spot Analysis**

We used hsFlp; FRT42D ubiGFP to generate loss-of-function clones of *cul-4* ExG1-3 and *cul-4* JH11 in the eye imaginal disc at different larval development stages. Egg layings were collected from synchronous cultures maintained at 25°C. The cultures were heat shocked at 24 and 48 hours after egg laying (AEL) at 37°C for 50 minutes in order to induce loss-of-function clones. Eye discs were dissected in second and third instar stages to detect *cul-4* loss-of-function clones that are marked by absence of GFP reporter {(Blair, 2003);(Sustar et al., 2011);(Xu and Rubin, 1993)}. The wild-type twin spots (strong GFP positive areas) of the respective clones
were marked and their intensities were measured using Adobe Photoshop 5.5 software and compared to the intensities of GFP negative areas (clones).

2.3 Immunohistochemistry

Eye-antennal imaginal discs were dissected from wandering third instar larvae and stained following the standard protocol (Singh et al., 2002). The eye imaginal discs were dissected in 1X solution of Phosphate Buffered Saline (PBS) and fixed using 4% p-formaldehyde (Electron Microscopy Sciences, Catalog no. 15710). Fixed tissues were washed using PBS-T {0.2% Triton X-100 (Sigma, Catalog no. T8787) in 1XPBS} and immunostained using specific antibodies (listed here below). Secondary antibodies (Jackson Laboratories) were goat anti-rat IgG conjugated with Cy5 (1:200), donkey anti-rabbit IgG conjugated to Cy3 (1:250), donkey anti-rabbit IgG conjugated to FITC, and donkey anti-mouse IgG conjugated to Cy3 (1:200), donkey anti-mouse IgG conjugated to FITC (1:250), and donkey anti-mouse IgG conjugated to Cy5 (1:250). The discs were mounted in Vectashield (Vector labs, H1000) and imaged using Olympus Fluoview 1000 microscope.

List of primary antibodies used:

Antibodies used were rat anti-Elav (1:100), mouse anti-Wg (1:50), mouse anti-β galactosidase (1:200), mouse anti-So (1:100), mouse anti-Dac (1:100), mouse anti-Eya (1:100), mouse anti-Dlg (1:200) (Developmental Studies Hybridoma Bank), rabbit anti-Dlg (1:250) (gift from Kyung-Ok Cho), rat anti-Mirror (1:200) (gift from Kwang Wook Choi), rabbit anti-caspase-3* (1:200) (Cell signaling Technologies), rabbit anti-Ey (a gift from Uwe Walldorf and Patrick Callaerts), anti-Hth (a gift from H. Sun and R. Mann), Rat anti-Tsh (1:50) (Gallet et al., 1998), chicken anti-GFP (1:200) (Upstate biotechnology), mouse anti-6E10 (1:100), (Kim et al., 1988) (Cell Science.com).
2.4 TUNEL Assay for Detection of Cell Death

Apoptosis was detected by using TUNEL assays (McCall and Peterson, 2004; Singh et al., 2006) (White et al., 1994). TUNEL assays are used to identify cells undergoing apoptosis where the cleavage of double and single stranded DNA is marked effectively. This protocol involves labeling DNA breakage by adding fluorescently labeled nucleotides to free 39-OH DNA ends in a template-independent manner using Terminal deoxynucleotidyl transferase (TdT). The fluorescein labels incorporated in nucleotide polymers can be detected by fluorescence microscopy. Eye-antennal discs, after secondary-antibody staining (Singh et al., 2002) were blocked in 10% normal goat serum in phosphate buffered saline with 0.2% Triton X-100 (PBT) for an hour. After blocking, samples were incubated in 0.1 M sodium citrate (Fisher Scientific) and 10% TritonX-100 for 30 minutes in dark at 65°C. Samples were washed and incubated in TUNEL dilution buffer (Roche Diagnostics, Catalog no. 11966006001) for 10 minutes followed by labeling with 50% TUNEL labeling solution for 30 minutes (Roche Diagnostics, Catalog no. 12156792910) in dark at room temperature. Samples were then subjected to TdT enzyme solution (Roche Diagnostics) for 2 hours at 37°C. Samples were then washed and mounted in Vectashield (Vector labs, Catalog no. H1000).

2.5 Western Blot Analysis

Protein samples were prepared from eye-antennal imaginal discs from third instar larvae of different cul-4 mutants in PBS and then subjected to boiling in sample buffer containing SDS-beta mercapto-ethanol for 10 minutes. Protein samples were separated on 10% SDS-PAGE and transferred to nitrocellulose membrane. Membrane was blocked in blocking solution (Amresco, Catalog no. M325-100) and subjected to incubation with anti- mouse Wg (DSHB); anti- mouse arm (DSHB), anti-rabbit p-JNK, anti-rabbit Caspase-9 (Cell Signaling Technologies) and anti-mouse tubulin (Sigma Aldrich) at a concentration of 1:100, 1:2000, 1:200, 1:1000 and 1:5000
respectively. Signal was detected using horseradish peroxidase-conjugated goat anti-rabbit IgG using super-signal chemiluminiscence substrate (Pierce, Catalog no.34076).

2.6 Scanning Electron Microscopy (SEM)

The fly samples for scanning electron microscopy (SEM) were prepared by dehydration through a series of gradient concentrations of acetone (Tare et al., 2009). Dehydrated flies were then transferred to 1:1 mixture of acetone and Hexa Methyl Di Silazane (HMDS, Electron Microscopy Sciences, Catalog no. 999-973), and then stored in 100% HMDS. The flies were allowed to air dry in HMDS. Dehydrated flies were mounted on a carbon conductive tape on EM stubs. Fly samples were coated with gold using a Denton vacuum sputter coater and analyzed using a Hitachi S-4800 High Resolution Scanning Electron Microscope (HRSEM).

2.7 Bright Field Imaging of the Eye

Adult flies were selected and were transferred to -20°C for two hours, followed by removal of legs and wings. The legs and wings were removed in order to get a clear view of the compound eye. The Bright field pictures of adult heads were taken using a Zeiss Apotome Imager Z1.
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specific gene expression during Drosophila melanogaster eye development. *Genetics* 141, 1075-86.


**FIGURE LEGENDS**

**Fig. 2.1:** A Schematic representation of strategy used for generating tissue specific knockout of gene function in genetic mosaics (also called as Loss-Of-Function, LOF clones). Mutant cells (−/−) clones were marked by absence of Green Fluorescent Protein (GFP) reporter (Black). Cells heterozygous for the mutation (−/+)) were marked by lighter green signal of GFP reporter (as they had a single copy of GFP). The wild type (+/+)) cells were marked by strong green signal of GFP reporter as they had two copies of GFP.
Fig. 2.2: In the GAL4/UAS system, the DNA binding GAL4 sequence restricts the expression of the target transgene that is cloned downstream to Upstream Activating Sequences (UAS). The multiple GAL4 binding sites in the UAS construct increase the expression level of the target gene several folds higher than the wild-type. Inset shows an eye imaginal disc expressing GFP (Green) transgene under the bi GAL4 promoter. bi drives expression of GFP (bi>GFP) reporter only on dorsal and ventral margins of the developing eye imaginal disc. Note that bi>GFP expression is also observed in dorsal antenna (Tare et al., 2013).
Higher Levels of Gene X in the tissues
CHAPTER 3
AN E3 UBIQUITIN LIGASE CULLIN-4 IS REQUIRED FOR CELL SURVIVAL FUNCTION IN THE VENTRAL EYE

Meghana Tare, Oorvashi Roy Puli, Shimpi Bedi, Madhuri Kango-Singh, Amit Singh

(Manuscript in preparation)
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In all multi-cellular organisms protein degradation is required during organogenesis to maintain protein homeostasis. However, the role of ubiquitin mediated protein degradation in dynamic regulation of asymmetric protein distribution during early eye development remains poorly understood. The aim of current work is to assign a role for an E3 ubiquitin ligase encoding gene, cullin-4 (cul-4 hereafter) during the early eye organogenesis in Drosophila melanogaster. There are previous reports which suggest involvement of cul-4 in controlling the cell cycle via ubiquitylation of different target proteins involved in cell cycle control. However, there are no reports indicating role of cul-4 in mediating cell survival via regulating asymmetric distribution of proteins during organogenesis. We have identified genetic function of cul-4 during eye development. Our studies indicate that E3 ubiquitin ligase Cul-4 is required during eye organogenesis to regulate a) Wg levels b) JNK levels in order to prevent cell death and promote cell survival.

Our studies provide multiple evidences for involvement of cul-4 in regulating asymmetric distribution of proteins in order to maintain protein homeostasis during the event of axial patterning during early stages of eye organogenesis.
INTRODUCTION

The compound eye of *Drosophila melanogaster* has been extensively used to study developmental processes like axial patterning and growth during organogenesis. Axial patterning involves generation of Dorsal-Ventral (DV), Antero-Posterior (AP) and Proximo-Distal (PD) axis, and is crucial for transition of a single sheet of cells into a three-dimensional organ. The compound eye of *Drosophila* develops from a sac-like epithelial structure called as eye-imaginal disc (Cohen, 1993; Held, 2002). In the larval eye imaginal disc a synchronous wave of retinal differentiation called the Morphogenetic Furrow (MF) moves from the posterior margin of the eye disc and leaves behind differentiated photoreceptor clusters called ommatidia (Ready et al., 1976; Wolff and Ready, 1991). The adult eye consists of about 750-800 ommatidia, which are arranged in mirror image symmetry along the DV axis.

The border between the dorsal and the ventral eye compartment is called the equator, which is the site for upregulation of Notch signaling to promote cell proliferation and differentiation (Baonza and Garcia-Bellido, 2000; Cagan and Ready, 1989; de Celis et al., 1996; Go et al, 2005). Thus, DV patterning, the first axis determination event in the developing *Drosophila* eye (Singh and Choi, 2003), is essential for cell proliferation and differentiation (Cho and Choi, 1998; Dominguez and de Celis, 1998; Papayannopoulos et al., 1998). The growth of dorsal and ventral domains in the eye disc is regulated differentially by domain specific interactions between wide networks of genes (Singh et al., 2005b; Singh et al., 2012). The early eye primordium has a default ventral state which requires function of Lobe (L), and a N ligand Serrate (Ser) (Singh and Choi, 2003; Singh et al., 2012). Loss of function of *L/Ser* results in the loss of ventral eye phenotype (Singh and Choi, 2003; Singh et al., 2005b; Singh
et al., 2012). The dorsal eye fate is established later by onset of expression of dorsal selector genes like GATA-1 transcription factor *pannier* (*pnr*) on the dorsal eye margin (Singh and Choi, 2003). The cell survival function of *SpnSer* in the ventral eye depends on the suppression of Wingless (Wg) signaling mediated induction of cell death in the eye (Singh et al., 2006).

Wg, a secreted morphogen and a ligand for the Wg signaling pathway binds to its transmembrane receptors Arrow (Arr) and Frizzled (Fz), and initiates an intracellular signaling cascade. In the absence of Wg signaling, β-catenin homolog Armadillo (Arm) is phosphorylated via Shaggy kinase (Sgg) and is degraded in the cytoplasm whereas Wg signaling facilitates transport of Arm to nucleus (Aberle et al., 1997; Archbold et al., 2011; Seto and Bellen, 2004). Thus, cytoplasmic versus nuclear levels of Arm decide the status of expression of Wg target genes. In the developing eye imaginal disc, *wg* is involved in several diverse functions of cell proliferation and cell death. Wg acts as a negative regulator of MF as loss-of-function of *wg* results in generation of ectopic furrows in both dorsal and ventral domains of the eye imaginal disc (Ma and Moses, 1995; Treisman and Rubin, 1995). Therefore, *wg* is required for defining the anterior regions of the eye disc which form the head capsule (Treisman and Rubin, 1995). During pupal development Wg is known to induce apoptotic genes *head involution defective* (*hid*), *reaper* (*rpr*) and *grim* to remove extra cells from the periphery of the pupal retina by triggering programmed cell death (Cordero et al., 2004; Lin et al., 2004; Ryoo et al., 2004; Singh et al., 2006). Inappropriate activation of Wg signaling in the eye imaginal disc also results in ectopic induction of cell death in the ventral eye (Singh et al., 2006).

In response to aberrant activation of signaling pathways and stress stimuli, cysteine proteases or Caspases get activated by cleavage of inactive zymogens, which results in induction of apoptotic cell death. During development cell death can occur due to caspase dependent and/or caspase independent mechanisms. The pro-apoptotic genes, *hid*, *rpr*, and *grim* are involved in the
intrinsic caspase-dependent cell death pathway (Chen et al., 1996; Grether et al., 1995; White et al., 1994). Apoptotic cell death is negatively regulated by inhibitors of apoptosis (IAPs). In *Drosophila* (DIAP-1) is responsible for negative regulation of *hid, rpr* and *grim* (Holley et al., 2002; Yoo et al., 2002). Inactivation of DIAP-1 due to proteolytic degradation can trigger caspase dependent cell death. In higher vertebrates cell death is caused by the activation of Caspase-3 and Caspase-9 (Drice and Dronic in *Drosophila*) (Chew et al., 2004; Daish et al., 2004; Xu et al., 2005). A baculovirus protein P35 can be used to block caspase-dependent cell death (Hay et al., 1994). Caspase-independent cell death is also regulated by the activation of c-Jun amino-terminal (NH$_2$) Kinases (hereafter JNK), which belongs to the MAP kinase super-family (Adachi-Yamada et al., 1999).

JNK signaling pathway is highly conserved across the species and is involved in cell proliferation and cell survival (Adachi-Yamada and O'Connor, 2004; Stronach, 2005). Activation by MAP kinases, intern activates JNK signaling which result in the initiation of a phosphorylation cascade. In *Drosophila*, JNK signaling is activated by Eiger (Egr), a homolog of Tumor necrosis factor (TNF) and its receptor Wengen (Wgn) by a conserved signaling cascade of Tak 1 (TGFb activating kinase 1), a JNK kinase kniase (JNKKK), hemipterous (hep) (JNK kinase), Basket (Jun kinase) and Jun. This results in activation of downstream target Puckered (Puc). Puc is a dual phosphatase and participates in a negative feedback loop by downregulating JNK activity (Davis, 2000). Activated JNK signaling in tissues causes caspase activation and results in induction of death (Adachi-Yamada et al., 1999; Adachi-Yamada and O'Connor, 2002; Moreno, 2008). Even though JNK signaling mediates cell death through rpr and hid, blocking caspase dependent cell death does not completely block JNK –dependent cell death which is also referred to as caspase-independent cell death.
In the ventral domain of the developing eye, cell death mediated by Wg is repressed by \( L \) but its exact mechanism is currently unknown. The mechanistic insight into this process will allow us to understand how ventral eye specific fate is established and maintained in the \textit{Drosophila} eye. It has been shown that DV patterning is initiated and established by domain specific function and expression of genes along the temporal axis. Thus, a dynamic regulation of domain specific localization of proteins is required for DV Patterning in the eye. We have identified \textit{culin-4} (\textit{cul-4} hereafter), which encodes an E3 ubiquitin ligase, as a ventral eye gene with domain specific function in \textit{Drosophila} eye. It belongs to evolutionarily conserved class of Cullin family of proteins that can utilize specific substrate recruitment proteins for different targets of destruction (Jackson and Xiong, 2009). It has been reported that \textit{cul-4} is involved in maintenance of genomic integrity by promoting the ubiquitylation and subsequent degradation of key regulators in cell cycle regulation (Dai and Wang, 2006; Higa et al., 2006b; Hu et al., 2008; Kim et al., 2006; Ou et al., 2003; Shibutani et al., 2008); (Korzelius and van den Heuvel, 2007). Collectively, all these previous reports suggest that \textit{cul-4} is involved in cell cycle regulation \textit{via} ubiquitylation of different substrates. However, role of \textit{cul-4} in regulating asymmetric gene expression to promote patterning in a developing field has not been studied.

Here, we report that \textit{cul-4} is required for generation of DV lineages in the developing eye. Further, \textit{cul-4} acts downstream of \( L \), and can modify \( L \) mutant phenotype in the ventral eye. In the developing eye \textit{cul-4} inhibits accumulation of Wg levels in the ventral domain to promote cell survival and facilitates cell proliferation. We present evidences to show that \textit{cul-4} prevents Wg signaling mediated caspase-dependent and JNK signaling mediated caspase independent cell death in the developing eye. Thus, we have identified a new role of \textit{cul-4} in cell survival independent of its functions in cell cycle regulation during eye development. Taken together, we
present evidences supporting a role for *cul-4* in regulating Wg activity for normal growth and survival of ventral eye cells.

**Material and Methods**

**Fly stocks:** Fly stocks used are described in Flybase (http://flybase.bio.indiana.edu). We used y w eyFLP (Newsome et al., 2000), EP 2518 (UAS-cul-4), y^1; P[SUPor-P]Cul-4^KGo2990/CyO; ry^506, which is a lac-Z reporter line of *cul-4* (Bellen et al., 2004). Mutant null alleles used in the study are *cul-4^{11L}/CyO-GFP* which lacks 65 amino acids from C terminal (Hu et al., 2008). N terminal deletion mutants used were *cul-4^{ExG1-3}/CyO*, which lack 340 amino acids from amino terminal. *cul-4^{11L}/twi>GFP, CyO* carries a non-sense mutation at Trp 199 position (Lin et al., 2009).

y w; L^{rev-3}FRT42D/CyO, L^2/CyO, L^si, (Chern and Choi, 2002; Singh and Choi, 2003), L^2/CyO; ey GAL4 (Singh et al., 2005a) were used in making double mutants of L with *cul-4*. Other stocks used in this study are *wg-LacZ/CyO* (Kassis et al., 1992), UAS-sgg^A9 (Hazelett et al., 1998), UAS-arm (Zecca et al., 1996), UAS-dTCF^DN (van de Wetering et al., 1997), UAS-P35 (Hay et al., 1994), Df(3L)H99/TM6B (Abbott and Lengyel, 1991), *puc^E69*, UAS-puc (Martin-Blanco et al., 1998), UAS-bsk^DN (Adachi-Yamada et al., 1999), and UAS-DJun^espv7 (Treier et al., 1995). GAL4/UAS system was used for targeted misexpression studies (Brand and Perrimon, 1993). We used *ey- GAL4* (Hazelett et al., 1998), and *bi-GAL4* (Calleja et al., 1996) for targeted misexpression studies.

**Mosaic Analysis**

To generate loss of function clones of *cul-4* in the eye, virgins of *eyFlp;FRT42D,CI-w^+*/CyO-GFP were crossed to i) FRT 42D, *cul-4^{ExG1-3}/CyO*, ii) FRT 42D, *cul-4^{ExG3-5}, iii) FRT 42D, *cul-4^{ExL2-1}/CyO* and, iv) FRT 42D, *cul-4^{11L}/twi>GFP, CyO.*
**Twin Spot Analysis**

We used *hsFlp; FRT42D ubiGFP* to generate loss-of-function clones of *cul-4<sup>EsG1-3</sup>* and *cul-4<sup>jj11</sup>* in the eye imaginal disc at different larval development stages. Egg layings were collected from synchronous cultures maintained at 25°C. The cultures were heat shocked at 24 and 48 hours after egg laying (AEL) at 37°C for 50 minutes in order to induce loss-of-function clones. Eye discs were dissected in second and third instar stages to detect *cul-4* loss-of-function clones that are marked by absence of GFP reporter [(Blair, 2003);(Sustar et al., 2011);(Xu and Rubin, 1993)]. The wild-type twin spots (strong GFP positive areas) of the respective clones were marked and their intensities were measured using Adobe Photoshop 5.5 software and compared to the intensities of GFP negative areas (clones).

**Immunohistochemistry**

Eye-antennal imaginal discs were dissected from wandering third instar larvae and stained following the standard protocol (Singh et al., 2002). Antibodies used were rat anti-Elav (1:100), mouse anti-Wg (1:50), mouse anti-β galactosidase (1:200) (Developmental Studies Hybridoma Bank), rabbit anti-Dlg (1:250) (gift from Kyung-Ok Cho), rat anti-Mirror (1:200) (gift from Kwang Wook Choi), rabbit anti-caspase-3* (1:200) (Cell signaling Technologies). Secondary antibodies (Jackson Laboratories) were goat anti-rat IgG conjugated with Cy5 (1:200), donkey anti-rabbit IgG conjugated to Cy3 (1:250), donkey anti-rabbit IgG conjugated to FITC, and donkey anti-mouse IgG conjugated to Cy3 (1:200). The discs were mounted in Vectashield (Vector Labs) and imaged using Olympus Fluoview 1000 microscope.
TUNEL Assays

Apoptotic cell death in the mutant clones generated via twin spot analysis and cell lethal approach was assayed using TUNEL assays. Eye discs after secondary antibody staining (Singh et al., 2002) were blocked in 10% Normal Goat Serum in Phosphate Buffered Saline with 0.2% Triton X-100 and TUNEL assays were done using the Cell-death Detection Kit from Roche Diagnostics following the standardized protocol (Singh et al., 2006; White et al., 1994).

Scanning Electron Microscopy (SEM)

The fly samples for scanning electron microscopy (SEM) were prepared by dehydration through ascending series of acetone concentrations (Tare et al., 2009). Dehydrated flies were then transferred to 1:1 mixture of acetone and Hexa Methyl Di Silazane (HMDS, Electron Microscopy Sciences), and then stored in 100% HMDS. The flies were allowed to air dry in HMDS. Dehydrated flies were mounted on a carbon conductive tape on EM stubs. Fly samples were coated with gold using a Denton vacuum sputter coater and analyzed using a Hitachi S-4800 High Resolution Scanning Electron Microscope (HRSEM).

Western Blot Analysis

Protein samples were prepared from eye-antennal imaginal discs from third instar larvae of different cul-4 mutants in PBS and then subjected to boiling in sample buffer containing SDS-beta Mercapto-ethanol for 10 minutes. Protein samples were separated on 10% SDS-PAGE and transferred to nitrocellulose membrane. Membrane was blocked in blocking solution (Amresco) and subjected to incubation with anti- mouse Wg (1:100) (DSHB); anti- mouse arm (1:2000) (DSHB), anti-rabbit p-JNK (1:2000) (Cell signaling Technologies), anti-rabbit Caspase-9 (1:1000) (Cell signaling Technologies) and anti- mouse tubulin (1:5000) (Sigma Aldrich). Signal
was detected using horseradish peroxidase-conjugated goat anti-rabbit IgG using super-signal chemiluminescence substrate (Pierce).

Results

cul-4 is a new member of the DV patterning pathway and acts downstream of L

In a forward gain-of-function genetic screen, we identified cul-4 as a modifier of L mutant phenotype of loss of ventral eye (Singh et al., 2005a). In comparison to the wild-type eye imaginal disc and the adult eye, which comprises of both dorsal and ventral halves (Fig. 3.1A, B), the L mutant eye shows selective loss of the ventral half as seen in the eye imaginal disc (Fig. 3.1C) as well as the adult eye (Fig. 3.1 D). In the adult eye the upper half of the eye is dorsal whereas the lower half is ventral (Fig.3.1 B). The loss of ventral specific eye in L mutant eye disc was detected by using localization of Mirror (Mirr), a member of Iro-C genes (Fig. 3.1A, C, E), which is a marker for dorsal-specific eye fate (McNeill et al., 1997). To analyze the genetic interactions between L and cul-4, we misexpressed cul-4 in the L mutant’s eye imaginal disc. We used an ey-Gal4 driver to misexpress cul-4 in the entire developing eye imaginal disc (Hazelett et al., 1998; Singh et al., 2005a). We found that misexpression of cul-4 can restore the L mutant phenotype of preferential loss of ventral eye (Fig. 3.1C, D) to a near wild-type eye imaginal disc (Fig.3.1 E) and the adult eye (Fig.3.1 F). These results suggest that cul-4 may act downstream of L in the ventral eye during early development. To validate this data, we analyzed the effect of L mutant background on cul-4 gene expression using a lacZ reporter line of cul-4 (Bellen et al., 2004). Prior to specification of the dorsal domain in the late first instar stage of larval development (Fig. 3.2 A, A’, eye primordium outline marked by yellow dotted line), cul-4 lacZ expression (red) is restricted to a domain of the developing eye primordium. However, after the
specification of the dorsal domain in the second instar stage (Fig. 3.2B, B’), *cul-4 lacZ* expression is restricted to the ventral half of the eye disc and nearly the entire antennal disc. At this stage there are no photoreceptors present in the eye imaginal disc. In the late third instar stage, when photoreceptor differentiation is completed (Fig. 3.2C, C’), *cul-4 lacZ* is preferentially expressed in the ventral half of the eye (arrow, expression domain outlined by dotted line) and anterior to morphogenetic furrow (Elav; blue), and in a few cells below dorsal head vertex region (near border of eye and antennal field marked by the white dotted line in Fig 3.2C’). This preferential ventral and dorsal vertex specific *cul-4* expression is also similar to expression of another ventral gene *fringe* (*fng*). *Fng* is preferentially expressed in the developing ventral eye disc and in the anterior dorsal vertex (Cho and Choi, 1998). The *cul-4 lacZ* expression is strongly downregulated in the ventral eye of *L\textsuperscript{2}* (Fig. 3.2 D, D’, white arrow) and *L\textsuperscript{rev}* (Fig. 3.2E, E’, white arrow) mutant backgrounds. These results further support that *cul-4* acts downstream of *L*. However, the nature of this genetic interaction is unclear. Therefore, we tested whether *L* and *cul-4* interact via synergistic or antagonistic mechanisms.

To address this question, we generated double mutants of *L* and *cul-4*. We first tested dominant interactions between *cul-4* and different *L* mutants (using their null and hypomorphic alleles) in the transheterozygotes. We reduced the levels of *cul-4* and *L* up to 50% in the double heterozygote using null alleles like *cul-4 ExG1-3* and *cul-4 JJ11* (Lin et al., 2009), and *L\textsuperscript{2}* and *L\textsuperscript{rev}* mutants (Chern and Choi, 2002). We found that upon reducing levels of *cul-4*, the loss of ventral half of the eye phenotype in the *L\textsuperscript{2}* mutant is enhanced to a “no eye” as seen in *L\textsuperscript{2}, cul-4 ExG1-3/+* (Fig. 3.3 A, B) and *L\textsuperscript{2}, cul-4 JJ11/+* (Fig. 3.3G, H). Reduction of *cul-4* levels in *L\textsuperscript{rev}* mutant background, a null allele of *L* (*cul-4 ExG1-3, L\textsuperscript{rev}+/+*), resulted in reduced eye size (Fig. 3C, D). The *L\textsuperscript{rev}/CyO* exhibits wild-type eyes in heterozygous combination (Chern and Choi, 2002). To rule out any allele specific interaction, we also tested other *L* alleles. *L\textsuperscript{s}* is a homozygous viable allele
of the \( L \), which exhibits a near wild-type eye in heterozygous background (Chern and Choi, 2002). When levels of \( \text{cul-4} \) were reduced in \( L^{st} \) heterozygous background (\( \text{cul-4}^\text{ExGl-3} \), \( L^{st}/+ \)), it resulted in the loss of ventral half of the eye (Fig.3.3E, F). These results suggest that \( L \) and \( \text{cul-4} \) act synergistically in the ventral eye, as individual mutant alleles did not show "no-eye" or highly reduced eye phenotype.

**Loss-of-function of \( \text{cul-4} \) results in preferential loss of ventral half of the eye**

We further characterized the effects of loss of \( \text{cul-4} \) function in the developing eye in somatic clones (Fig. 3.4). We tested various \( \text{cul-4} \) alleles that encode Cul-4 protein lacking different regions from C and N terminus (Fig. 3.4A). We employed the \( \text{ey-Flippase} \) (\( \text{ey-flp} \)) or heat shock-flp (\( \text{hs-flp} \)) mediated mitotic recombination approach to generate genetic mosaic clones of \( \text{cul-4} \) in the eye (Blair, 2003; Xu and Rubin, 1993). We differentially marked \( \text{cul-4}^{\text{+/+}} \) cells (by loss of GFP) and wild-type twin spot cells (GFP homozygous), and measured clone sizes in eye discs. The \( \text{cul-4} \) loss-of-function clones generated by traditional heat shock approach in first or second instar larvae could not be recovered in third instar eye disc or the adult eye, as only wild-type twin-clones that express 2Xubi-GFP were seen. Furthermore, no phenotypes were observed in the eye disc as well as the adult eye (data not shown). In the dorsal eye few clones (GFP- negative) are observed but they were very small and comprised of only a few cells (data not shown). However, large wild-type sister clones marked by strong GFP reporter were observed. Together these data revealed that the \( \text{cul-4} \) mutant cells are eliminated sometime before the wandering third instar stage, and suggested that \( \text{cul-4} \) mutant clones may be eliminated due to competitive interactions between the mutant and wild-type cells. To test cell proliferation profiles of \( \text{cul-4} \) mutant cells, we did ‘twin-spot analysis’. We used heat shock-flippase to induce mitotic recombination in the first instar and examined \( \text{cul-4} \) loss-of-function early in the second and third
instar stage to see whether these clones can survive. Unlike the controls where we generated wild-type clones using the GFP chromosome (Fig. 3.4B), the \textit{cul-4} mutant cells could not be recovered in the third instar eye disc where clones were induced by heat shock at 24h of development. To observe the effects of loss of \textit{cul-4} in mutant cells before they are eliminated, we induced the mutant clones at later time points (48h or 60h of larval development). The clones generated by these strategies revealed smaller \textit{cul-4}\textsuperscript{−/−} clones, (Fig. 3.4C; clone boundary marked by red dotted lines) at 96h of development (data not shown). However, these clones were eliminated at 120h of development. Quantification of mutant clone areas and their twin spots showed that \textit{cul-4} mutant clones are significantly smaller than their wild-type twin clones (Fig. 3.4C). This effect was much more pronounced in the ventral eye (Fig. 3.4C). Together, these data suggest that \textit{cul-4} mutant clones may be slow growing, and out-competed by the surrounding wild type cells.

We then employed cell lethal approach to study loss-of-function of \textit{cul-4} in the eye imaginal disc where wild-type cells are eliminated (Newsome et al., 2000). Using the cell lethal approach, we created a condition where, nearly 80\% of cells in the eye imaginal disc lack \textit{cul-4} function. This resulted in the preferential loss-of-ventral eye phenotype in the eye imaginal disc as well as the adult eye (Fig.3.4E). Loss of function of \textit{cul-4}\textsuperscript{ESG1:3} (Lin et al., 2009) resulted in preferential loss of ventral eye as evident from expression of dorsal fate marker Mirr in dorsal half of the eye and significant reduction in the ventral half of the eye (Fig. 3.4 E). In wild-type eye imaginal disc Mirr is expressed in the dorsal half of eye disc (Fig. 3.4D). However in \textit{cul-4}\textsuperscript{ESG1:3} clones the ventral half of the eye disc marked by absence of Mirr expression is highly reduced compared to the dorsal half (Fig. 3.4E). Loss- of-function clones of \textit{cul-4}\textsuperscript{H111} (Lin et al., 2009), exhibit reduction of the ventral compartment both in the eye disc (Fig. 3.4F) as well as the adult eye (Fig. 3.4G). Our expression profile and loss-of-function studies indicated that \textit{cul-4}, acts downstream to \textit{L} and exhibits DV constraint in its expression, as well as the function. \textit{L} has been
shown to promote ventral eye survival by repressing Wg signaling in the ventral eye during early eye developmental stages (Singh et al., 2006). Therefore, we next tested interactions of cul-4 and wg.

**cul-4 represses Wg levels in the ventral eye disc**

To test if Wg levels are affected by cul-4, and if cul-4 acts upstream or downstream of Wg, we first tested Wg expression levels in L and cul-4 mutant background. In the third instar stage, Wg is expressed along the antero-lateral margins of the wild-type eye disc (Fig. 3.5A, A'); Baker, 1988). Wg expression in the dorsal domain is to establish growth of dorsal domain of the eye (Maurel-Zaffran and Treisman, 2000; McNeill et al., 1997) whereas in the ventral Wg is required to suppress eye fate and define the ventral boundary of the eye (Ma and Moses, 1995). In L and cul-4 double mutant (L^2, cul-4^{Eg1-3/+}) which exhibits highly reduced eye field (Fig. 3.3 A) robust ectopic induction of Wg was observed (Fig. 3.5B-5B'). It is known that L downregulates Wg signaling in the ventral eye (Singh et al., 2006). To rule out the possibility that Wg induction in L and cul-4 double mutant is only due to loss of L function, we tested the expression of Wg in cul-4 mutant background. We generated loss-of-function clones of cul-4 using cul-4^{Eg1-3} (Fig. 3.5C, C') and cul-4^{J111} (Fig. 3.5D, D') mutant allele, and found a robust ectopic induction of Wg in the eye disc (Fig. 3.5C', D') along with reduced ventral half of the eye. We isolated total protein extracts for cul-4^{Eg1-3} and cul-4^{J111} mutant eye imaginal discs and analyzed levels of Wg protein. We found that Wg is expressed at higher levels were in the cul-4 mutant backgrounds as compared to wild-type eye disc (Fig. 3.5E). Together this data from genetic interactions and semi-quantitative western blots suggests that cul-4 is involved in down regulation of Wg in the eye imaginal disc. Wg serves as a ligand for the Wg signaling in the eye. We found that misexpression of Wg using bi-Gal4 driver results in ectopic wg transcription in the eye. This
suggests that \(wg\) serves as one of the downstream targets of Wg signaling in the eye (Fig. 3.10). Therefore, we next analyzed the effects of loss and gain of Wg signaling on \(cul-4\) mutant phenotypes.

**Canonical Wg signaling pathway alters the \(cul-4\) mutant phenotype**

It is known that Wg, prevents the cytoplasmic phosphorylation and proteolysis of cytoplasmic transducer Arm. As a result, a cytoplasmic pool of Arm is generated which enters the nucleus and turns the transcription of Wg target genes “on” via nuclear transcription factor dTCF (Seto and Bellen, 2004); Fig. 3.6A). First, we analyzed the levels of Arm protein, in \(cul-4\) mutant eye discs and observed that Arm levels are up-regulated in \(cul-4\) loss-of-function condition as compared to the wild-type levels of Arm (Fig. 3.6B). Next, we tested if \(cul-4\) mutant phenotype is affected by modulating the levels of Wg signaling pathway. Misexpression of \(wg\) in the developing eye imaginal disc (\(ey\textgreater wg\)) results in reduced eye phenotypes (Fig. 3.6C). Misexpression of \(wg\) in eye imaginal disc where entire eye disc cells are mutant for \(cul-4\) function (\(cul-4^{-}\), \(ey\textgreater wg\)) using cell lethal clonal approach (Newsome et al., 2000), resulted in near complete elimination of the eye (detected by loss of expression of photoreceptor specific marker ELAV or loss of ommatidia) in the eye disc and adult eye respectively (Fig. 3.6D, E). Misexpression of \(arm\) in eye imaginal disc (\(ey\textgreater arm\)) results in highly reduced eye (Fig. 3.6F). In \(cul-4\) loss-of-function background misexpression of \(arm\) (\(cul-4^{-}\), \(ey\textgreater arm\)) resulted in highly reduced or no-eyes (Fig. 3.6 G, H). We also tested the effect of blocking Wg signaling on \(cul-4\) loss-of-function phenotypes. Shaggy/Zeste-White-3/GSK-3 is a negative regulator of Wg signaling pathway (Heslip et al., 1997); (Treisman and Rubin, 1995). Misexpression of \(sgg\) in the eye imaginal disc (\(ey\textgreater sgg\)) does not affect the size of eye field (Fig. 3.6I). However, increasing
levels of sgg by overexpression in *cul-4* loss-of-function background (*cul-4*<sup>−/−</sup>, *ey*<sup>sgg</sup>) resulted in rescue of *cul-4* loss-of-function phenotype to a normal eye in the eye disc as well as the adult eye (Fig. 3.6 J, K). The most downstream component of Wg signaling, TCF belongs to TCF/LEF family of transcription factor (van de Wetering et al., 1997). Misexpression of dominant negative form of TCF (*dTCF<sup>DN</sup]*) (van de Wetering et al., 1997) in eye disc (*ey* > *dTCF<sup>DN</sup>*<sup>−/−</sup>) results in normal eye imaginal disc (Fig. 3.6L). Misexpression of *dTCF<sup>DN</sup>* in *cul-4* loss of function background (*cul-4*<sup>−/−</sup>, *ey* > *dTCF<sup>DN</sup>*<sup>−/−</sup>) resulted in rescue of *cul-4* loss-of-function phenotype to a near wild type eye disc and adult eye (Fig.3.6 M, N). Given that reduction of Wg signaling levels can rescue *cul-4* loss-of-function phenotype, it suggests that (a) *cul-4* is involved in downregulation of Wg in the eye, (b) *cul-4* acts upstream of *wg* in the eye disc. Ectopic Wg signaling is known to cause cell death in the developing eye (Cordero et al., 2004; Lin et al., 2004). Thus, it is possible that the phenotypes observed in *cul-4* mutant cells may result from inappropriate activation of cell death due to accumulation of high levels of Wg. We therefore, investigated if the *cul-4* loss-of-function phenotype is linked to Wg mediated induction of ectopic cell death.

**Cul-4 promote cell survival in the ventral eye**

To test if ectopic Wg expression is responsible for inducing cell death in *cul-4* loss-of-function clones, we first checked if caspases were activated in *cul-4* mutant cells. In *cul-4* loss-of-function clones we found strong induction of activated Caspase-3* (Cas-3*) staining along with the induction of Wg (Fig. 3.7A, A’, A”). The Cas-3* staining was more robust in the ventral domain of the eye disc. We tested protein levels of Caspase-9 in the total protein extracted from the eye imaginal disc of wild type as well as eye tissues mutants for *cul-4*. We found that caspase-9 levels were nearly three folds higher in the *cul-4* mutant background (Fig. 3.7B). Thus, loss-of-function of *cul-4* in the eye disc results in ectopic induction of Wg in the ventral eye, resulting in
cell death, as marked by presence of up-regulated Cas-3* Over-expression of baculovirus protein P35 selectively blocks Caspase-dependent cell death (Hay et al., 1994). Overexpression of P35 in *cul-4* loss-of-function clones generated by cell lethal approach resulted in approximately 60% rescue of the eye size (Fig. 3.7C-C”, D). However, in these discs, we were still able to see up-regulated Wg levels (Fig. 3.7C’). During development cell death is caused by the pro-apoptotic genes *hid, reaper* and *grim* (collectively called as HRG complex). We reduced the levels of HRG complex by using a deficiency (H99) that uncoverts all the three genes in the *cul-4 loss-of-function* background and observed that preferential loss of ventral eye is rescued to 100% (Fig. 3.7E, E”, F) and Wg levels are downregulated that are comparable to wild-type Wg levels (Fig. 3.7E’). Overall, these data suggest that *cul-4* loss-of-function causes up-regulated Wg signaling which results in preferential ventral eye loss due to induction of cell death.

**cul-4 cell survival function is mediated through JNK Signaling.**

Our earlier observation that *cul-4* mutant cells are slow growing suggests that the elimination of *cul-4* mutant cells could be due to cell competition. This lead us to analyze JNK levels in *cul-4* mutant cells as the JNK signaling pathway is implicated in the regulation of cell proliferation and cell survival (Adachi-Yamada and O’Connor, 2004; Stronach, 2005). The downstream target of the JNK signaling pathway is (*puc*), a dual phosphatase, which serves as the functional read-out for the activation of the pathway (Martin-Blanco et al., 1998). We checked the expression of *puc* to test if JNK signaling pathway is affected in *cul-4* mutant background. Puc is expressed in the differentiated photoreceptor neurons in eye disc (Adachi-Yamada, 2002), Fig. 3.8A). In *cul-4* mutant background, we observed a strong ectopic induction of *puc* in the ventral half of the eye as well as the antennal field (Fig. 3.8B, B’). This suggests that JNK signaling is activated in *cul-4* mutant cells. To validate our results, we tested levels of p-JNK protein in tissue extracts of eye disc having *cul-4* mutant clones generated by cell lethal approach.
and found that p-JNK levels are up-regulated in *cul-4* mutant eye discs compared to the wild-type eye disc tissue extracts (Fig. 3.8B). We asked if the mutant phenotype of *cul-4* can be altered by changes in levels of JNK signaling. First, we tested if blocking JNK signaling in the *cul-4* mutant background can rescue the *cul-4* mutant phenotype of loss of ventral eye. We blocked JNK signaling using misexpression of *puc* and *bsk* dominant negative (Adachi-Yamada et al., 1999) in the eye disc where *cul-4* loss-of-function clones are generated by cell lethal approach. Misexpression of *puc* in wild type conditions, does not affect the eye size (Fig. 3.8D) whereas, misexpression of *puc* in the eye disc where *cul-4* loss-of-function clones are generated by cell lethal approach, resulted in significant restoration of mutant phenotype of loss of ventral eye (Fig. 3.8, E, F). Misexpression of a dominant negative form of *bsk* (*bsk^{DN}*) does not affect the eye size (Fig. 3.8J). Misexpression of *bsk^{DN}* in the eye disc where *cul-4* loss-of-function clones are generated by cell lethal approach resulted in restoration of *cul-4* mutant phenotype of loss of ventral eye to a near wild-type eye (Fig. 3.8K, L).

Conversely, activation of JNK signaling pathway by misexpression of activated Jun (*jun^{asp7]*) in the eye disc where *cul-4* loss-of-function clones are generated by cell lethal approach, strongly enhanced the mutant phenotype of loss of ventral eye to a “no-eye” (Fig. 3.8 H, I). Misexpression of *jun^{asp7}* alone in the eye results in highly reduced eye field (Fig. 3.8G). These data suggest that *cul-4* mutant cells are rendered vulnerable to competitive interactions mediated by induction of Wg and activation of JNK signaling. Thus, *cul-4* may be involved in limiting JNK activation in the developing eye disc, especially in the ventral eye cells and thereby promoting their survival during development.
Discussion

During organogenesis in multi-cellular organisms, axis generation is a lineage restriction event which leads to generation of different compartments. The compartments are the fundamental units of patterning and growth. The genetic machinery required for delineation of compartment specific fate is crucial for patterning and growth. The genetic mechanisms controlling generation of compartments are highly conserved across the species. This process of compartment generation in the *Drosophila* eye begins with the delineation of DV axis and depends on several genetic clues. In the eye, DV axis determination requires domain specific expression of dorsal genes and domain specific function of ventral eye genes (Singh et al., 2012). Proper expression and turnover of proteins in the dorsal or ventral cells is required for orchestrating the events that will culminate in the generation of dorsal and ventral specific cell fates and their differentiation.

Genetic machinery involved in protein degradation machinery has been known to play role in protein homeostasis during development. The genetic machinery involved in ubiquitin mediated protein degradation has been shown to be involved in organogenesis, growth, differentiation, metabolism and ageing in all the organisms (Varshavsky, 2012). Several genes that encode proteins required for degradation via proteosomal pathways have been identified, and mutations in these genes interfere with proper patterning, differentiation and growth of eye. Ubiquitination is an important process, required for transcriptional and post-translational modifications of proteins. In *Drosophila* eye, an established model to study facets of cell biological processes during development and growth, members of ubiquitination process has been shown to regulate cell cycle, patterning and growth. A variety of E3 ligases like tumor suppressor protein Archipelago (Ago) (Nicholson et al., 2009), Neuralized (Neu), a component of Notch signaling pathway (Cho and Fischer, 2011), Mind Bomb (Mib), another E3 ubiquitin ligase, are
present in the developing *Drosophila* eye and are required for proper eye morphogenesis (Nagaraj and Banerjee, 2009). Another ubiquitin ligase Slimb has been shown to be involved in regulating Wg and Hh signaling in the eye during development (Jiang and Struhl, 1998). Cul-1 and Cul-3 have been shown to regulate Ci stability in the developing *Drosophila* eye (Ou et al., 2002).

*Drosophila* eye develops from a default ventral state and *L* is responsible for cell survival in the ventral half of the eye (Singh and Choi, 2003; Singh et al., 2006). We have identified in a forward genetic screen that *cul-4* act as a modifier of *L* mutant phenotype of loss of ventral eye (Fig. 3.1). Our genetic epistasis studies suggest that *cul-4* acts downstream of *L* (Fig. 3.1, 3.2), and both *L* and *cul-4* act synergistically in ventral eye development (Fig. 3.3).

**cul-4 is a new DV patterning gene**

In this study we present multiple evidences suggesting that the E3 ubiquitin ligase *cul-4* is a new DV patterning gene that is involved in axial patterning of the developing *Drosophila* eye. Our studies strongly suggests a new function of *cul-4* in the development of ventral compartment of the eye, *cul-4* is expressed in ventral cells and is required for cell survival of ventral cells in the developing eye. Characterization of *cul-4* loss of function alleles revealed that *cul-4* mutant cells are slow growing and eliminated from the disc epithelium by the neighboring wild-type cells via activation of JNK and Caspase mediated intrinsic cell death pathway. Overall, our studies place *cul-4* as a new member of the hierarchy of DV patterning genes, that acts downstream of *L* and is involved in the regulation of Wg expression in the ventral domain of the *Drosophila* eye.

We looked into the mechanism by which *cul-4* promote ventral eye development. We have shown that *L* is involved in downregulating Wg in the ventral eye (Singh et al., 2006). These
studies provided evidence that downregulation of Wg signaling by $L$ is mediated through $cul-4$ gene (Fig. 3.5). In the third instar eye-antennal imaginal disc, $wg$ expression is seen both on anterior and lateral margins of dorsal and ventral domains (Fig. 3.5A, A’). In the double heterozygotes of $L$ and $cul-4$, we tested the Wg levels and found that the Wg levels are up-regulated as compared to wild-type Wg levels (Fig. 3.5B, B’). This suggests that $cul-4$ is involved in DV patterning by regulating Wg levels.

**Loss of $cul-4$, a ventral eye gene, shows preferential loss of ventral eye**

Using a $lacZ$ insertion line we found that $cul-4$ has domain specific expression (Fig. 3.2). We employed genetic mosaic approach to discern the role of $cul-4$ in DV patterning of the developing *Drosophila* eye. When we used the classical genetic mosaic approach where cells lacking $cul-4$ function are marked by absence of GFP reporter in the eye, we were not able to see the loss-of-function clones of $cul-4$ in the ventral eye. However, the dorsal eye clones were also smaller than their wild-type twin spot (Fig. 3.4B). These studies suggested that $cul-4$ mutant cells are probably eliminated. The twin spot analysis further suggested that wild-type twin spot clones marked by strong GFP reporter showed robust growth. These results also suggested that $cul-4$ mutant cells are possibly eliminated due to cell competition. To analyze $cul-4$ function we generated $cul-4$ loss-of-function clones using the cell lethal strategy (Newsome et al, 2002). In these eye imaginal discs where all the wild-type twin-spot clones were eliminated by cell lethal mutation, we observed preferential loss-of-ventral eye phenotype. We verified this ventral eye loss using the dorsal fate specific markers (Fig. 3.4). These results suggested that $cul-4$ mutant cells are eliminated in the presence of wild-type cells.

**Cell lacking $cul-4$ function are eliminated by cell competition**
Loss of function analysis of *cul*-4 mutant cells revealed that *cul*-4 mutant cells are slow growing and tend to be eliminated by surrounding wild-type cells. The growth rate of both the dorsal and ventral specific clones is slower than their wild-type twin spots (as seen by the comparison of twin-spot analyses in the two compartments. It is noteworthy that the *cul*-4 mutant clones in the ventral eye are more vulnerable or are eliminated earlier than their dorsal counterparts, indicating that the sensitivity of cells to loss of *cul*-4 function is different for the dorsal and ventral eye. This may be because Cul-4 is expressed in the ventral and therefore, loss of *cul*-4 more predominantly affects *cul*-4 functions in the ventral eye. The elimination of the mutant clones from the developing eye discs suggested that cell competition may be involved in the interactions of *cul*-4 mutant cells and their wild-type neighbors. Cell competition is a phenomenon where cells compare their fitness and the less fit cells are eliminated by induction of cell death by their more fit neighbors. The activation of cell death during cell competition is known to occur via activation of the JNK pathway. Indeed, analysis of *cul*-4 mutant clones for activation of JNK target genes (*puc*) confirmed this interaction. In addition, activated Caspases (*Cas*) were observed in *cul*-4 mutant clones preferentially in the ventral domain. It has been shown that cells lacking Wg activity survive, whereas, cells with hyperactive Wg signaling are eliminated (Vincent et al, 2011). Our studies of interaction of *cul*-4 and Wg support the hypothesis since Wg signaling is up-regulated in *cul*-4 mutants.

***cul*-4 mutant cells are lost by induction of cell death**

Since ectopic Wg is known to induce developmental cell death, we tested the role of *cul*-4 in preventing cell death in the eye. The ventral eye cells mutant for *cul*-4 were eliminated due to induction of cell death as evident from our caspase stainings (Fig 3.7). We found that *cul*-4 also promotes ventral eye survival by preventing ectopic Wg signaling mediated cell death in the
developing *Drosophila* eye. We verified the induction of developmental cell death due to loss of *cul-4* function by rescuing the *cul-4* mutant phenotypes by blocking caspase mediated cell death (Fig. 3.7C, D). However, the rescues were not complete as a result we also tested the caspase independent cell death in the *cul-4* mutant clones.

**Cell survival the novel function of *cul-4* in the developing eye**

Previously, Cul-4 has been shown to be involved in variety of processes including maintenance of genomic integrity by promoting the ubiquitylation and subsequent degradation of key regulators in cell cycle regulation (Dai and Wang, 2006; Higa et al., 2006a; Hu et al., 2008; Kim and Kipreos, 2007; Korzelius and van den Heuvel, 2007; Shibutani et al., 2008). The Cul-4 DDB1 complex of ubiquitin ligase has been shown to mediate polyubiquitylation of many crucial regulator of the eukaryotic cell cycle. Recent reports on involvement of Cul-4 in degrading cell cycle regulators has generated a perspective of its role, in general, into growth regulatory mechanisms which include various cell signaling pathways as well.

Therefore, when we generated loss-of-function clones of *cul-4* we expected that these clones will show growth defects. Surprisingly, when we tried to see the fate of these cells where *cul-4* function was abolished, we found that cell lacking *cul-4* function are eliminated by induction of cell death. Our results demonstrated that this cell death is mediated through Wg signaling as blocking the Wg signaling could restore the loss of ventral eye phenotype seen in the *cul-4* loss-of-function. We have provided evidences for involvement of Cul-4 in cell survival mechanisms during early stages of development preferentially in the ventral eye. Earlier reports have shown that axial patterning genes control growth and survival of the cells in domain specific manner (Singh et al., 2012). Through this study, we outlined a mechanism that *cul-4* a component
of protein degradation machinery is instrumental in controlling ventral specific function in gene function in developing *Drosophila* eye (Fig. 3.9).

Any disturbance in protein degradation pathway could lead to various disorders ranging over growth defects to neurodegenerative disorders (Dennissen et al., 2012). We have provided evidence for L mediated cell survival function of *cul-4* in developing *Drosophila* eye which shares genetic similarities with that of vertebrate eye. The ubiquitin mediated protein degradation via *cul-4* of different target proteins has already been reported for genomic integrity and cell cycle regulatory mechanisms. In the developing eye, it will be interesting to find more targets of *cul-4* function which might be involved in generation of axes.

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Fig.3.1 *cul-4*, a ubiquitin ligase, may act downstream of *L*. (A) Wild-type eye-antennal imaginal disc showing expression of a membrane specific marker, Dlg (red); dorsal eye fate marker Mirr (green); and a pan-neural marker Elav (blue), (B) and the adult eye. (C, D) The preferential loss of ventral eye phenotype as seen in *L* mutant (C) eye imaginal disc as evident from dorsal eye fate marker expression, (D) and adult eye. The white dotted straight line marks equator, the boundary between the dorsal and the ventral eye compartment. (E, F) Misexpression of *cul-4* in the entire developing eye field in the *L* mutant background (*L2; ey> cul-4*) results in the significant rescue of the *L* mutant phenotype of loss-of-ventral-eye to a near complete. The ventral eye rescue in eye disc is based on domain of Mirr, a dorsal eye fate specific marker expression.
**Fig. 3.2 cul-4 expression is preferentially ventral.** Spatio-temporal profile of *cul-4* expression during eye imaginal disc development exhibits a domain specific pattern. (A-A’) *cul-4* expression (red) in first instar eye disc is restricted to a domain in the eye disc. (B-B’) *cul-4* expression evolves and gets restricted to the ventral eye domain in the second instar eye disc. Note that there is no retinal differentiation in the eye at this stage as is evident from the Elav (blue) expression. (C-C’) *cul-4* expression is restricted anterior to the morphogenetic furrow (MF) in a group of cells both ventral (arrow) and dorsal (arrowhead) eye domain. *cul-4* transcription is reduced in absence of *L* (D, E). Double mutant eye antennal imaginal disc of *L^{rev}* (D) or *L^{2}* (E) and *cul-4 lac-Z* line. *cul-4* expression is highly reduced in *L* mutant background (Arrows in D’ and E’).
**Fig.3.3 cul-4 interacts genetically with L.** Double mutants of L and cul-4 result in enhancement of eye phenotypes compared to L and cul-4 individual mutants. Eye-antennal imaginal disc (A) and SEM of adult head (B) for double heterozygote of $L^2$ and $cul-4$ $^{ExG1-3}$ mutant, stained for membrane marker Dlg and pan-neural marker Elav. Note that Elav staining is completely lost in the eye imaginal disc, indicating that double mutant of L and cul-4 results in no eye phenotype, which seems like an additive effect, since $L^2$ mutation results in loss of photoreceptor cells from just ventral half of the eye. Eye antennal imaginal disc of double mutant of $L'^{av}$ (A null and FRT recombed allele of L and has a WT eye by itself), and $cul-4$ $^{ExG1-3}$ (C) and scanning electron micrograph of adult head (D) indicates loss of photoreceptor cells from eye field as compared to individual mutants. Double mutant of $cul-4$ with $L'^{d}$ mutant shows loss of eye field (E, F). $cul-4^{jjj}$ is a null allele of $cul-4$ with a non-sense mutation at W199 position, when recombined with $L^2$ allele, shows loss of the entire eye field in the imaginal disc (G) (absence of Elav staining in D); adult head structure lacks any ommatidia in scanning electron micrograph (H).
Fig. 3.4 Loss-of-function of *cul-4* results in the preferential loss of ventral eye. (A) Schematic representation of wild-type Cul-4 protein and proteins encoded by different mutant alleles of *cul-4* used in the loss-of-function studies. (B) Control showing GFP positive and negative patches created using Flp-FRT system. Note that clone areas, marked by absence of GFP (red dotted lines), are comparable to wild-type strong GFP positive areas (marked by red dotted lines). (C) Loss-of-function clone for *cul-4*JJ11 mutant in the eye imaginal disc (marked by red dotted line) are smaller compared to wild-type twin spot (marked by yellow dotted line). So, *cul-4* mutant cells have growth defects in the eye. (D) Wild-type eye imaginal disc stained for dorsal specific marker Mirr (green), membrane marker Dlg (blue) and pan neuronal marker Elav (red). (E) Loss-of-function clone of *cul-4* created using cell lethal technique results in preferential loss of ventral eye as seen in the eye imaginal disc stained for dorsal specific marker Mirr (green) and neuronal marker Elav (Red). Note that only ventral eye is lost upon *cul-4*ExG1-3 loss-of-function, dorsal eye stays normal as seen by expression of Mirr in dorsal. *cul-4*JJ11 allele also shows similar phenotypes of loss-of-ventral eye (E) in the disc and in the adult eye, (F). Other mutant alleles of *cul-4* were also tested and they show similar phenotypes of loss of ventral eye (data not shown).
**Fig. 3.5 Cul-4 suppresses Wg in the ventral eye.** Loss of *cul-4* results in the preferential loss-of-ventral-eye along with the up-regulation of Wg. Heterozygous combination of *L* and *cul-4* mutant allele result in ectopic Wg induction and preferential loss-of ventral eye as seen in (B,B’) eye antennal disc for *cul-4<sup>ExG1-3</sup>* and *L<sup>2</sup>* double mutants, compared to (A,A’) wild-type eye imaginal discs. Abolishing levels of *cul-4* in the eye disc using loss-of-function approach causes ectopic induction of Wg staining in (C,C’) *cul-4<sup>ExG1-3</sup>* and (D,D’) *cul-4<sup>JJ11</sup>* mutants. (E) Protein levels of Wg are higher in mutant discs of *cul-4* as compared to Wild-type levels of Wg, tested using Western Blotting analysis.
Fig. 3.6 Canonical Wg pathway alters the cul-4 mutant phenotype. (A) Cartoon showing canonical Wg pathway. Wg is a signaling morphogen which upon binding to its receptors on membrane, causes transcription of target genes via its cytoplasmic transducer Arm and nuclear target TCF. (B) Protein levels of Armadillo (Arm), a downstream cytoplasmic target of Wg signaling are enhanced compared to WT, as seen on western blot performed using protein extract prepared from loss of function of cul-4 eye-antennal imaginal discs. (D, E) Enhancing the levels of Wg pathway by increasing levels of wg, (G, H) arm results in enhancement of loss-of-function phenotype of cul-4. Misexpression of wg (C), arm (F) reduces the eye size compared to wild-type eye discs. Reducing levels of Wg signaling by increasing levels of negative regulators of the pathway Sgg (I, K) and dTCF\textsuperscript{DN} (M, N) suppresses the ventral eye loss phenotype of cul-4 and restores the eye size, close to normal eye. Misexpression of sgg (I) and dTCF\textsuperscript{DN} (L) in the eye results in normal eye sizes.
Fig.3.7 up-regulated Wg signaling due to loss-of-function of *cul-4* results in caspase dependent apoptotic cell death. Loss-of-function of *cul-4* in the eye antennal imaginal disc results in ectopic induction of Wg in the ventral eye, resulting in cell death. Eye antennal imaginal disc stained for Cleaved caspase-3 (cas-3*, green), Wg (red) and neuronal marker Elav (blue). (A, A’, A”’) *cul-4* loss-of-function results in enhanced caspase-3* levels along with up-regulated Wg signaling. (B) Protein levels of activator caspase-9 are higher in loss-of-function clones of *cul-4* in the eye imaginal discs as compared to wild-type levels of caspase-9. (C, C’, C”’)

The ventral eye loss resulted due to loss-of-function of *cul-4* can be rescued by over-expression of baculoviral protein P35 in *cul-4* mutant background. Eye-antennal imaginal disc stained for membrane marker Dlg (green), Wg (red) and Elav (blue), shows rescue of *cul-4* loss of function by overexpressing P35 in the eye imaginal disc and the (D) adult eye. (E, E’, E”’) Simultaneous reduction in the levels of Hid-Reaper-Grim complex (by using deficiency of H99) along with loss-of-function of *cul-4* rescues the preferential ventral eye loss phenotype, as seen in the eye imaginal discs and the (F) adult eye.
Fig. 3.8 Induction of JNK pathway in loss-of-function of *cul-4*. (A,) *puc-lacZ* is expressed in differentiated photoreceptor neurons posterior to morphogenetic furrow in wild-type eye imaginal discs (Adachi Yamada, 2002). (B, B’) Upon loss-of-function of *cul-4* in the eye, *puc-lacZ* is strongly induced onto ventral margins of the eye disc. (C) Blocking JNK signaling by misexpression of *puc* doesn’t affect eye size in wild-type condition (D), but misexpressing *puc* in loss-of-function clones of *cul-4* rescues the *cul-4* mutant phenotype of ventral eye loss in the eye imaginal disc and the adult flies (E, F). Overexpression of activated form of *jun* in wild type condition reduces the eye size (G), whereas, (H, I) overexpression of activated *jun* along with *cul-4* loss-of-function situation results in enhancement of ventral eye loss phenotype. Misexpression of dominant negative form of *bsk* in wild type condition in the wild-type eye results in no effect on eye size (J), whereas, (L, L) overexpressing dominant negative *bsk* in *cul-4* loss-of-function condition restores the ventral eye loss to a great extent.
Fig. 3.9 Model illustrating mechanism of cell survival function of *cul-4* in the eye. We have shown that *cul-4* acts downstream of L. Cul-4 acts downstream to L in order to i) block Wg signaling to prevent Wg mediated, caspase dependent cell death, and; ii) inhibit JNK signaling to prevent Caspase independent JNK mediated cell death. Thus, in this study, we show that *cul-4* functions in promoting cell survival during early stages of eye organogenesis.
Fig. 3.10. Wg serves as its own target in the eye imaginal disc. Wg transcription is ectopically induced upon misexpression of wg. Using a domain specific Gal4 (bi), we misexpressed UAS wg transgene onto dorsal and ventral margins of the eye imaginal disc and tested transcription of wg using wg lac-Z (red) line. Along with reduction in eye size on dorsal and ventral sides, (marked by absence of Elav, A”), we observed that wg transcription was ectopically induced on the dorsal and ventral margins of the eye (A’).
$bi \succ wg$

A  Dlg  A'  wg-Z  A''  Elav
CHAPTER 4

DORSAL EYE SELECTOR PANNIER (Pnr) SUPPRESSES THE EYE FATE TO DEFINE DORSAL MARGIN OF THE DROSOPHILA EYE

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The understanding of genetic interactions and hierarchy controlling event of axial patterning during *Drosophila* eye development is far from complete. Axial patterning is one of the crucial events for organogenesis involving dorsal-ventral (DV), anterior-posterior (AP) and proximal-distal (PD) axes. This event is important for i) Growth regulation in the eye, ii) transformation of single layer of cells into a three dimensional organ structure; and iii) correct targeting of axons from retina to the brain; (discussed in details in literature review section).

*Drosophila* eye primordium develops on a default ventral state over which dorsal boundary is specified upon expression of a GATA-1 transcription factor *pannier* (*pnr*, hereafter). Dorsal eye margins are further defined by induction of Wg by *pnr* which in turn is required for activation of Iro-C genes (Iroquious-Complex comprising aracua, capaulican and mirror); the dorsal markers in the eye.

The current work provides mechanistic insights for suppression of eye by *pnr* in order to define the dorsal boundary of the developing eye field. We have found that Loss-of-function of *pnr* results in dorsal eye enlargements where as misexpression of *pnr* results in the loss of eye field.

Our studies indicate that in the early eye developmental stages, *pnr* is required for assigning dorsal eye fate, whereas, in later stages, *pnr* suppresses the retinal determination by downregulation of homeotic gene *tsh*, and is independent of Meis class gene *hth*. 
Dorsal eye selector *pannier* (*pnr*) suppresses the eye fate to define dorsal margin of the *Drosophila* eye

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Fig. 4.1. Pnr expression is restricted to the peripodial membrane (PM) of the dorsal eye margin. (A) pnr expression (pnr Gal4 drive UAS-GFP, Singh and Choi, 2003; Singh et al., 2005) is absent in the first instar eye-antennal imaginal disc whereas Wg (red) is expressed in the entire eye disc. Note that pnr expression in the brain at this stage is seen. (B) In the second instar eye-antennal imaginal disc, pnr expression (green) is initiated in 15-20 cells on the dorsal eye margin and Wg (red) is expressed laterally on both dorsal and ventral eye margins. At this stage, Hth (blue) expression is present in the entire eye disc. (C, C, C”) In the early third instar eye-antennal disc, pnr expression in the dorsal eye margin is restricted only to the peripodial membrane (PM) whereas Hth (blue) is also expressed in peripodial membrane (PM) of the eye-antennal disc. (C’) pnr (green) expression at this stage is absent in the disc proper (DP). Hth (blue) expression begins to retract with the initiation of MF and stays anterior to the furrow (Pai et al., 1998; Bessa et al., 2002; Singh et al., 2002). (C”) Pnr expression is restricted to the peripodial membrane (PM) specific cells on the dorsal eye margin. (D) In the late third instar eye-antennal imaginal disc, pnr (green) expression is restricted to the dorsal eye margin whereas Wg (red) expression is restricted to the dorsal and ventral eye margins. Hth (Blue) is expressed in rings in the proximal region of antenna and expressed both in the dorsal and ventral part of the disc proper anterior to the furrow. Dashed lines indicate the approximate midline, the border between D (Dorsal) and V (ventral) eye. All the eye-antennal imaginal discs and the adult eyes are organized as Dorsal (D) up and the ventral (V) down. Markers for immunostaining are shown in color labels. (AN: Antenna).
Fig. 4.2. Loss-of-function of *pnr* exhibits a range of eye enlargements and antennal duplications in the dorsal eye. (A, B) Loss-of-function clones of *pnr* in the dorsal eye margin {marked by the absence of GFP (green) in the eye-antennal imaginal disc and absence of the mini-white reporter (red) in the adult eye} results in a non-autonomous ectopic eye enlargement as seen in the eye-antennal imaginal disc and in the adult eye. The ectopic eye enlargements are not restricted within the clone. However, they extend both in the wild-type as well as in the *pnr* mutant cells of the eye-antennal disc. Note that the dorsal clone boundary is marked by white dotted line in the eye disc and by black dotted line in the adult eye. (C, D) Loss-of-function of *pnr* in the dorsal eye results in an autonomous ectopic dorsal eye anterior to the normal eye field. These ectopic eyes are restricted to within the clones. Note that not all the cells of the *pnr* loss-of-function clone differentiate to the photoreceptors. (E, F) Loss-of-function clones of *pnr* in the dorsal eye have no effect on the eye field as seen in the eye disc and the adult eye. All these clones were restricted to the disc proper. (G, H) Loss-of-function clones of *pnr* in the antenna results in duplication of the antennal field as seen in (G) the eye-antennal disc and (H) the adult head. (H) Scanning electron microscopy (SEM) of the adult head showing antennal duplication and dorsal eye enlargement (Magnification X180). Note that only a few *pnr* loss-of-function clones show both dorsal eye enlargements along with the antennal duplication.
Fig.4.3. Pnr suppresses the eye fate. (A) Eye-antennal imaginal disc showing domain of expression of GFP reporter (green) under the ey Gal4 (ey>GFP). Note that the ey Gal4 drives the expression of GFP reporter in the entire eye-antennal imaginal disc (both anterior as well as posterior to the morphogenetic furrow (MF) marked by white arrowhead). (B) Wild-type adult eye. (C, D) Misexpression of pnr in the eye using the ey-Gal4 driver (ey>pnr^{D4}) results in the suppression of eye fate and leads to a “no-eye” phenotype in the (C) eye-antennal disc (Elav, a pan-neural marker, which marks the photoreceptors) as well as in the (D) adult eye. The white dotted line in 3C marks the possible outline of the eye disc. There is no effect on the antennal field both in the eye-antennal imaginal disc as well as the adult head. (E) Another Gal4 driver, bi-Gal4 drives expression of a GFP reporter (bi>GFP) both on the dorsal and the ventral eye disc margin. (F) Misexpression of pnr using bi-Gal4 (bi>pnr^{D4}) results in the suppression of eye fate on both the dorsal and the ventral eye margin as evident from the loss of Elav expression (white arrows). (G, G’, H) Gain-of-function clones of pnr (marked by GFP, white arrowhead) generated by random “flp-out” approach in the eye using the heat shock-FLP showed the suppression of eye as evident from the absence of (G’) Elav in the eye disc as well as (H) in the adult eye. Note that the eye suppression in the pnr heat shock “flp out” clones was seen only in the larger clones. Further, necrosis (black spots) is also seen in the adult eye upon misexpression of pnr in the eye. (I, J) Blocking pnr function in the entire eye using pnr^{ENR} construct (ey>pnr^{ENR}) results in a “small eye” phenotype as seen (I) in the eye imaginal disc as well as (J) in the adult eye. (K) However, blocking pnr function both on the dorsal and the ventral eye disc margin (bi>pnr^{ENR}) results in the dorsal eye enlargement whereas there was no effect on the ventral eye margin. This data suggests that pnr suppresses eye on the dorsal eye margin.
Fig. 4.4. Pnr suppresses the expression of retinal differentiation genes in the eye. (A, A', A'"
Loss-of-function clones of pnr in the eye exhibit dorsal eye enlargement by (A'") ectopic Elav expression. In these clones where dorsal eye enlargement is seen, (A') the expression of retinal precursor marker Ey is restricted anterior to the furrow (white arrow). The dorsal eye enlargement, marked by Elav (blue) is the outcome of the pnr loss-of-function clone. Note that Ey is absent in the differentiating photoreceptors. Therefore, Ey is not seen in these clones. (B-D) Loss-of-function clones of pnr showing an ectopic dorsal eye phenotype with ectopic induction of retinal determination genes like (B, B', B'"") Eya (white arrow), (C, C', C'"") So (white arrow), and (D, D', D'"") Dac (white arrow). Note that these retinal determination genes, which act downstream to Ey, and unlike Ey are expressed in the differentiating photoreceptor neurons. (E- H) Gain-of-function of pnr in the eye suppresses the retinal determination genes. (E, E') Misexpression of pnr on dorsal and ventral eye margins by using a bi-Gal4 driver (bi>pnr<sub>D4</sub>), results in strong upregulation of Ey on both dorsal and ventral eye margins (marked by a white arrowhead). (F-H) However, misexpression of pnr (bi>pnr<sub>D4</sub>) suppresses the downstream retinal differentiation genes (F, F') Eya, (G) So, (H) Dac on both dorsal and ventral margins (marked by arrow heads). The anterior Dac expression (anterior to furrow) went all the way down to the posterior margin in bi>pnr<sub>D4</sub> misexpression. Ey marks retinal precursor cells and is required for the specification of eye field. Our results suggest that pnr may not affect early eye specification function of Ey, whereas pnr suppresses the retinal determination genes like eya, so and dac, which acts downstream to Ey.
**Fig. 4.5. pnr induces downstream target Wg to suppress the eye.** Wg is known to act as a negative suppressor of eye fate. Wg is expressed laterally both on the dorsal and the ventral eye margins (Fig. 1B). (A, A’) Misexpression of pnr on both dorsal and ventral eye margin using bi-Gal4 results in the suppression of eye on both DV margins along with ectopic induction of Wg (marked by white arrows). (B) bi>pnrD4 results in the reduction of eye both on the dorsal and ventral eye margins. This phenotype is similar to bi>wg (Singh et al., 2002). (C) Misexpression of Wg in the entire eye using ey-Gal4 (ey>wg) results in “no-eye”. (D) Loss-of-function clones of pnr in the dorsal eye (marked by absence of GFP reporter and white dotted line) result in the ectopic eye enlargement along with the suppression of Wg expression. (E, E’) Loss-of-function clones of pnr in the DP (marked by white dotted line) caused no effect on Wg expression as Pnr is not expressed in the DP. (E’) Higher magnification of the clone showing its location restricted to the DP. (F) Interestingly, some of the bigger loss-of-function clones of pnr (marked by white dotted line) exhibit the dorsal eye enlargement. However, this eye enlargement do not cover the entire clone. The part of the clone anterior to the eye enlargement show robust Wg expression. These clones show some overgrowth in the eye disc, which will form head specific structures, suggesting that within dorsal eye margin pnr is not the sole Wg regulator.
Fig. 4.6. *pnr* suppresses the eye fate at dorsal eye margin independent of *hth*. *hth*, a Meis class of gene (Rieckhof et al., 1997), acts as a negative regulator of the eye (Pai et al., 1998). Hth expression is restricted anterior to furrow in 10-15 cell wide domain and in entire peripodial membrane (Fig. 1). (A, A’) Misexpression of *hth* on both dorsal and ventral eye margin (*bi>*hth) results in the suppression of eye fate on both dorsal and ventral margin of the eye disc as evident from (A’) suppression of Elav (marked by white arrows). (B, B’) Misexpression of *pnr* on both dorsal and ventral eye margin (*bi>*pnr<sup>D4</sup>) results in suppression of eye on both dorsal and ventral eye margin (marked by white arrows), which is accompanied by induction of Wg (green) as well as Hth (red; white arrows). (C, C’) Loss-of-function clone of *hth* in the eye has DV asymmetric phenotypes. The loss-of-function clone of *hth* in the ventral eye results in the eye enlargement as evident from Elav expression (marked by white dotted line). Note that the dorsal eye clones do not exhibit any phenotype. (D- F) In loss-of-function clones of *pnr*, (D, D”) which result in dorsal eye enlargement (marked by white dotted line) or (E, E’) which do not exhibit dorsal eye enlargement (marked by white dotted line), Hth (red) expression stays anterior to the furrow as seen in the wild-type eye disc. Loss-of-function clones of *pnr* in the antennal disc which results in the duplication of antennal field exhibit ectopic Hth expression in the duplicated antennal disc (marked by white dotted line). Note that *hth* is expressed in the proximal region of the antennal disc.
Fig. 4.7. Pnr suppresses the eye fate by downregulating teashirt (tsh) in the dorsal eye margin. Tsh, a Hox gene (Fasano et al., 1991), exhibits Dorso-ventral (DV) asymmetric function in the eye (Singh et al., 2002). *pnr* expression initiates in early second instar eye-antennal imaginal disc (Singh & Choi, 2003). (A) In the late second instar, *pnr* expression evolves and is restricted to 50-100 cells of the dorsal eye margin. At this stage when MF has just initiated, Tsh is expressed anterior to the furrow (MF). (B) In the third instar eye imaginal disc, *pnr* is expressed on the dorsal eye margin whereas *tsh* is expressed in the eye disc anterior to the furrow. (C–C") Loss-of-function clone of *pnr* in the dorsal eye marked by the loss of GFP reporter (marked by white dotted line) exhibit (C') ectopic localization of Tsh protein, and (C'”) ectopic expression of tsh reporter (*tsh^{AB}/CyO*) in the dorsal eye. (D-D'”) Loss-of-function clones of *pnr* (marked by loss of GFP), where *tsh* function is reduced to half using a heterozygous background of *tsh* null allele (*tsh^{+/+}*), (D’) exhibit outgrowth on the dorsal eye margin which is positive for Ey expression but there is no ectopic eye enlargement as evident from (D””) absence of neuronal marker Elav expression. The dorsal overgrowth exhibits robust expression of Ey, a marker for undifferentiated retinal precursor cells. (E) Loss-of-function clone of *pnr* in the *tsh* heterozygous background marked by the loss of mini-white reporter (red: clonal boundary marked by black dotted line) results in the absence of eye enlargement in the adult eye. These results suggest that *pnr* eye suppression function is mediated through down regulation of *tsh*. (F) Misexpression of *pnr* on the dorsal and the ventral eye margin, *bi>pnr^{D4}* , results in the suppression of *tsh* reporter on both dorsal and ventral eye margin (white arrowhead) along with the suppression of eye as evident from Elav (blue) expression. Note that eye size is reduced on both margins. (G) Misexpression of *pnr^{D4}* both on dorsal and ventral eye margin in *tsh* heterozygous background (*tsh^{+/+}; bi>pnr^{D4}* ) exhibits strong suppression of eye resulting in a highly reduced eye. Note that *bi>pnr^{D4}* alone (F) shows suppression of eye both on the dorsal and the ventral eye margin. However, the size of *bi>tsh^{+/+}; pnr^{D4}* eye imaginal disc size is extremely reduced as compared to *bi>pnr^{D4}* alone. (H)
Misexpression of $tsh$ on DV margin ($bi>tsh$) results in the suppression of eye on the ventral margin whereas eye enlargement in the dorsal eye (Singh et al., 2002). Misexpression of both $tsh$ and $pnr$ on DV margin results in early lethality. Therefore, we misexpressed $pnr$ downstream target $ara$ with $tsh$. (I) Misexpression of $tsh$ with dorsal eye selector $ara$, a downstream target of $pnr$, on DV margin using $bi$-Gal4 ($bi>tsh+ara$) results in the enlargement on both dorsal and ventral eye margins. Misexpression of $tsh$ and $ara$ on DV margin results in strong dorsal eye enlargements. (J) Misexpression of $tsh$ using $pnr$-Gal4 driver ($pnr>tsh$) results in the enlargement of the dorsal eye. (K) Misexpression of $tsh$ in the heterozygous $pnr$ background results in the dorsal eye enlargement. However, these eye enlargements are not bigger than what is seen in (H) $bi>tsh$ or (J) $pnr>tsh$, suggesting that $pnr$ acts upstream of $tsh$. 
Fig. 4.8 Pnr suppresses the eye fate by downregulating *tsh* which results in suppression of retinal determination genes at the dorsal eye margin. GATA-1 transcription factor *pnr*, which is expressed in the peripodial membrane (PM) at the dorsal eye margin, suppresses the retinal determination. The suppression of retinal determination genes by *pnr* can be mediated by two possible ways: (i) *pnr* directly suppresses the retinal determination genes to suppress the eye. *pnr* may act downstream to *ey* and suppress the downstream retinal determination target *eya* and other downstream genes *so* and *dac*. During eye development, *ey* is required for eye specification and other downstream targets are required for retinal determination. Our studies suggest that *pnr* acts on retinal determination process, which corresponds to the onset of *pnr* expression in the eye. (ii) Alternatively, *pnr* suppresses the eye by downregulating homeotic gene *teashirt* (*tsh*) in the dorsal eye. Interestingly, the *tsh* gain-of-function in the dorsal eye (Singh et al., 2002) is complementary to the loss-of-function of *pnr* in the dorsal eye. *tsh* is known to act upstream of *eya*, *so* and *dac* (Pan and Rubin, 1998). Thus, the dorsal eye enlargement observed in *pnr* mutant is due to ectopic induction of *tsh* in the dorsal eye, which in turn can induce the RD genes. Lastly, Pnr mediated suppression of the eye fate is independent of Meis class of homeotic gene, *homothorax* (*hth*) function.
CHAPTER 5

ACTIVATION OF JNK SIGNALING MEDIATES AMYLOID-β-DEPENDENT CELL DEATH

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The neurocrystalline lattice, an often referred term for *Drosophila* retina enables it to be used as an ideal model for studying several developmental and neurological diseases. In previous chapters, we have mentioned that defects in axial patterning during organogenesis of the eye leads to developmental defects. This chapter however focuses on use of *Drosophila* eye as a model system to study Aβ42 accumulation mediated neuronal cell death.

Alzheimer’s disease (AD) is characterized by dementia, inability to perform simple routine tasks and results because of neuronal cell death. Neuronal cell death during the course of disease is caused due to accumulation of Aβ42 formed due to incorrect cleavage of amyloid precursor protein (APP). The precise reason leading to this incorrect cleavage of APP is not yet known. Current work describes how we developed *Drosophila* eye as a model system to study the Aβ42 accumulation mediated neuronal cell death leading to Alzheimer’s disease. We also found that out of several signaling pathways, c-Jun NH (2)-terminal kinase (JNK) pathways are actively involved in cell death caused by Aβ42 accumulation.

Our studies using *Drosophila* eye as a model system thus identify a key factor involved in AD neuropathology which can be used as a target for therapies.
Activation of JNK signaling mediates Amyloid-β-dependent cell death

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**Fig.5.1. Misexpression of amyloid-beta 42 (Aβ42) induces cellular phenotypes in the differentiating retinal neurons.** (A, A’) GMR-Gal4 drives expression of GFP reporter (GMR>GFP) in the differentiating photoreceptor neurons in the third instar eye imaginal disc. (A’) Magnified view of GMR>GFP expression domain. (B, B’) Misexpression of Aβ42 (GMR>Aβ42) restricts the Aβ42 expression (green channel) to the differentiating photoreceptor neurons as detected using 6E10 antibody (red channel) in eye imaginal disc. (C, E) Normal eye development in (C, C’, C”) third instar larval eye imaginal disc, (E, E’) early pupal (white pupa) retina. (D, F) Misexpression of Aβ42 (GMR>Aβ42) results in the induction of cellular phenotypes in the (D) developing third instar larval eye disc and (F) early pupal retina. Disc large (Dlg, red channel) marks the membrane, Elav (green channel), a proneural marker that marks the neuronal fate. (D, D’, D”) In the third instar larval stage, morphological changes are not as severe but exhibit slight “holes” (arrows) as a result of Aβ42 plaque accumulation. (F) In early pupal retina, there is an increase in disarray throughout the eye field. (F’) The ommatidial clusters are clumped or fused together (arrows, outlines of ommatidial clumps marked by dotted line), (E) unlike the wild-type ommatidia.
Fig. 5.2. Aβ42 accumulation in the developing pupal retina and adult eye results in neurodegeneration. (A, C) represent normal eye development in (A) late pupal retina, and (C) the adult compound eye. Dlg (red channel) marks the membrane; 6E10 (blue channel) marks the Aβ42, and Elav (green channel), a proneural marker that marks the neuronal fate. (B, D, F) Misexpression of Aβ42 in the differentiating neurons of eye using a GMR-Gal4 construct (GMR> Aβ42) results in onset of neurodegeneration. (C, C’) Unlike the highly organized ommatidia of wild-type pupal retina, (D, D’) the late pupal retina shows significant neuronal cell death in the eye field. By this stage, the accumulation of Aβ42 plaques has resulted in distinct holes in the eye field (marked by an arrow, outline of “hole” in pupal retina marked by red dotted line). (C) Wild type adult eye with uniform arrangement of 800 unit eyes. (I) The adult eye field of GMR> Aβ42 is significantly diminished and there is complete fusion of the ommatidia. (G, H) In comparison to the adult eye section of (G) wild type fly eye, (H) the GMR > Aβ42 exhibits highly disorganized morphology of photoreceptors. Furthermore the retinas of GMR> Aβ42 are vacuolated. This illustrates how the neurodegenerative phenotype progressively worsens as the age and dose increase over the course of fly development.
Fig.5.3. Misexpression of Aß42 triggers cell death in the differentiating neurons. (A, A’)
Wild-type third instar larval eye imaginal disc displaying randomly distributed TUNEL positive
dying cells (A, A’’) shown in red channel (arrow). Note that TUNEL staining marks the
fragmented DNA within the nuclei of dying cells [29,32,61]. (B) Wild-type adult eye. (C, C’)
Misexpression of Aß42 (GMR>Aß42) in differentiating neurons of the eye show elevated levels
of TUNEL positive cells (C’ arrows). The increased frequency of cell death in neurons can be
directly correlated to the misexpression of the Aß42 peptide. Note that misexpression of Aß42
does not affect the differentiation process as the distribution of Elav positive cells is the same in
both control and Aß42 third instar eye imaginal discs. (D) GMR>Aß42 results in a strong
neurodegenerative phenotype in adult eye. Baculovirus P35 has been shown to block the caspase
dependent cell death [36]. (E, E’, F) Misexpression of P35 along with Aß42 in differentiating
neurons (GMR>Aß42+ P35) shows significant reduction of dying cells based on number of
TUNEL positive cells (red channel) in the larval eye field. However, this rescue is not as strong
in (F) adult eye phenotype. (E’) Note that the eye field displays reduced number of TUNEL
positive cells (arrow) compared to GMR>Aß42 eye field (C’). It is important to note that Aß42
peptide production is not affected. Elav marks the photoreceptor fate (C’’’). Puckered (Puc), a
dual phosphatase, is downstream target of JNK signaling pathway and forms a feedback loop to
negatively regulate the pathway [20]. (G, G’, H) Misexpression of puc along with Aß42 in the
differentiating neurons (GMR>Aß42+ puc) results in significant suppression of cell death as
evident from reduced number of TUNEL positive cells in the third instar larval eye imaginal disc
as well as in the (H) adult eye. Note that there is a significant rescue of (D) GMR>Aß42 adult eye
phenotype in (H) GMR>Aß42+ puc background. These results suggest that JNK signaling might
be responsible for neurodegeneration seen in amyloid plaque mediated cell death. (I)
Quantification of the number of dying cells in eye imaginal discs based on TUNEL staining in
wild-type (served as control), GMR>Aβ42, GMR>Aβ42+ P35 and GMR>Aβ42+ puc. Note that blocking JNK signaling (GMR>Aβ42+ puc) exhibit strong rescue of the neurodegenerative phenotype of GMR>Aβ42 and GMR>Aβ42+ P35. This rescue is significant (**) as seen by calculation of P-values based on one-tailed t-test using Microsoft Excel 2007.
Fig. 5.4. **JNK signaling is activated upon misexpression of Aβ42 in the eye.** (A) Schematic representation of hierarchy of Jun-kinase signaling pathway members. (B, B’) Wild-type expression of *puc* in the developing third instar larval eye imaginal disc using lacZ reporter where reporter (red channel) is restricted only to the developing photoreceptors in the eye disc proper and in the peripodial membrane cells on the margin of the antennal disc [43]. (C, C’) GMR>Aβ42 eye imaginal disc exhibits ectopic upregulation of *puc-lacZ* reporter. (C’) Split channel showing ectopic *puc-lacZ* expression in the photoreceptor neurons of the eye imaginal disc. (D) Activation of JNK signaling in GMR>Aβ42 was detected by checking phospho-Jun levels. Levels of JNK signaling pathway increases three fold in GMR>Aβ42 as compared to the wild-type eye imaginal disc.
A

Wild-type

B, B'

puc-Z
Elav

GMR>Aβ42

C, C'

puc-Z
Elav

D

CS
pJNK
Tubulin
Fig. 5.5. Ectopic upregulation of JNK signaling induces cell death in the GMR>\textit{A\beta42} eye imaginal disc. (A) Wild-type eye imaginal disc showing cell death in random cells, which serve as controls. (B) GMR>\textit{A\beta42} eye imaginal disc showing ectopic upregulation of \textit{puc} lacZ in a large number of dying retinal cells as evident from TUNEL positive staining. In comparison to the (C) wild-type adult eye, (D) GMR>\textit{A\beta42} adult eye are highly reduced due to neurodegeneration. (E-L) Increasing level of JNK signaling in GMR>\textit{A\beta42} by misexpressing (F, H) activated \textit{hemipterous} (GMR>\textit{A\beta42+ hep}^{Act}) and (J, L) activated \textit{Djun} (GMR>\textit{A\beta42+ jun}^{asp\gamma}) results in (F, J) dramatic increase in dying cell population in the eye imaginal disc, leading to a (H, L) “no-eye” phenotype in the adult fly. Increased levels of (E, G) activated \textit{hemipterous} (GMR>\textit{hep}^{Act}), (I, K) activated \textit{Djun} (GMR>\textit{jun}^{asp\gamma}) served as controls and result in some dying cells in the (E, I) eye imaginal disc and (G, K) a small adult eye. However, reducing level of JNK signaling in GMR>\textit{A\beta42} background by misexpressing (N, P) Dominant negative \textit{basket} (GMR>\textit{A\beta42+ bsk}^{DN}) results in significant reduction to near complete absence of dying cell population (N) in the eye imaginal disc, leading to a (P) strong rescue of the adult eye phenotype as compared to GMR>\textit{A\beta42} adult eye. (M, O) Increased levels of Dominant negative \textit{basket} (GMR>\textit{bsk}^{DN}) in (M) eye imaginal disc and (O) adult eye served as controls. Note that increased levels of dominant negative \textit{basket} alone (GMR>\textit{bsk}^{DN}) does not affect the size of eye imaginal disc and the adult eye.
Fig. 5.6. JNK signaling is responsible for cell death in GMR>AB42. (A, A’) Misexpression of both P35 and puc along with AB42 (GMR>AB42+ P35 + puc) results in strong rescue of cell death as evident from (A’) dramatically reduced TUNEL positive cells. However, the rescue of the phenotype was not significantly stronger than with blocking JNK signaling pathway alone (Figure 5G’). (B) Misexpression of AB42 (GMR>AB42) in pupal retina showing cell death as evident from TUNEL positive cells (red channel). Blocking simultaneously both caspase-dependent cell death and caspase-independent JNK signaling mediated cell death in pupal retina (GMR>AB42+ P35 + puc) showed a strong rescue in (C, C’, C”) pupal retina and (D) adult eye as compared to (B) GMR>AB42 pupal retina, (Figure 3D) GMR>AB42 adult eye. The cell death is detected by TUNEL staining (red channel), which is (C’, C”) restricted to the periphery of the pupal retina. Note that dying cells on the periphery of the pupal retina corresponds to the programmed cell death as seen in the wild-type pupal retina too [25,28]. (E) Quantification of the number of dying cells in eye imaginal discs based on TUNEL staining in different genetic combinations. The frequency of cell death in wild-type eye imaginal disc served as a control. Note that blocking JNK signaling (GMR>AB42+ puc) or blocking JNK signaling along with caspase-dependent cell death (GMR>AB42+ P35+ puc) exhibit strong rescue of the neurodegenerative phenotype of GMR>AB42. This rescue is significant (**) as seen by calculation of P-values based on one-tailed t-test using Microsoft Excel 2007.
Domain Specific Genetic Mosaic System in the Drosophila Eye

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Summary: Genetic mosaic approach is commonly used in the Drosophila eye by completely abolishing or mis-expressing a gene within a subset of cells to unravel its role during development. Classical genetic mosaic approach involves random clone generation in all developing fields. Consequently, a large sample size needs to be screened to generate and analyze clones in specific domains of the developing eye. To address domain specific functions of genes during axial patterning, we have developed a system for generating mosaic clones by combining Gal4/UAS and flippase (FLP)/FRT system which will allow generation of loss-of-function as well as gain-of-function clones on the dorsal and ventral eye margins. We used the bifid-Gal4 driver to drive expression of UAS-FLP. This reagent can have multiple applications in (i) studying spatio-temporal function of a gene during dorso-ventral (DV) axis specification in the eye, (ii) analyzing genetic epistasis of genes involved in DV patterning, and (iii) conducting genome wide screens in a domain specific manner. genesis 51:68–74, 2013. © 2012 Wiley Periodicals, Inc.

Key words: Drosophila eye; Dorso-Ventral axis; patterning; genetic mosaic; Gal4/UAS technique; compartments

Technologically, researchers have exploited the ability to create genetic mosaics to circumvent the problems of lethality associated with studies to analyze function of essential genes. In Drosophila, the genetic mosaic techniques, where animals containing a distinct population of somatic cells lacking the function of a gene, have been used to address many basic biological questions including restriction of cell fates during patterning and growth (Golic, 1991; Xu and Rubin, 1993; Duffy et al., 1998; Lee and Luo, 1999; Stowers and Schwarz, 1999; Newsome et al., 2000; Lee and Luo, 2001; Blair, 2003). Several other approaches were developed that allowed the gain-of-function as well as the loss-of-function of two different genes in the same set of cells, for example, MARCM system (Lee and Luo, 1999, 2001). The development of genetic tools and reagents over the last three decades has vastly facilitated the analysis of gene function along the spatial and temporal axis. Majority of the genetic components of known pathways have been identified but the crosstalk among the various established pathways is yet to be understood. To test and validate the crosstalk among the pathways in a specific context of time, space or domain, it is important to develop simple but effective strategies to study the genetic hierarchy using genetic epistatic approaches. Thus, there is need to develop new tools which can be used to test both loss-of-function as well as gain-of-function of two different genes in a subset of cells within a developing field.

Genetic mosaic approach has been used in the Drosophila eye to address many biological questions includ-
ing study of cell fate, patterning, growth, cell death, and disease. There are several genetic mosaic approaches available in *Drosophila* (Golic and Lindquist, 1989; Golic, 1991; Xu and Rubin, 1993; Lee and Luo, 1999; Stowers and Schwarz, 1999; Newsome et al., 2000; Lee and Luo, 2001; Blair, 2003). *Drosophila* eye has also been used for genome wide screens which exploit the flippase (FLP) recombinase from the yeast to generate FRT-mediated recombination to screen for the genes affecting patterning, growth, death, and disease (Duffy *et al.*, 1998; Newsome *et al.*, 2000; Blair, 2003; Janody *et al.*, 2004; Call *et al.*, 2007; Morante *et al.*, 2011). The FLP enzyme catalyzes recombination at sequence motifs termed FLP-recombinase target sequences (FRTs). The heat shock mediated FLP expression to generate random clones may not be suitable in several instances as it may cause lethality due to the fact that extensive numbers of clones are produced (Duffy *et al.*, 1998). The approach of generating random clones to discern the gene function has proved to be extremely useful; however, in case of domain specific analysis using this approach requires sampling of a large number of clones. Most of these strategies provide excellent tools for generation of clones in the entire developing eye field. During organogenesis, a developing field gets further subdivided into smaller regions called compartments (Blair, 2001; Curtiss *et al.*, 2002; Dahmann *et al.*, 2011; Singh *et al.*, 2012). The properties of the cells of a compartment are unique and the cells within a compartment behave differently based on their response to the morphogen gradients. Therefore, to investigate the fine tuning of gene functions in the developing fields, it is important to design tools that will facilitate knocking down a gene in a subset of cells in a developing field or compartment.

We have combined the Gal4/UAS system used for targeted misexpression (Brand and Perrimon, 1993) with the FLP/FRT system (Evans *et al.*, 2009) to generate genetic mosaics. In our study, we have employed an *optomotor blind* (omb) or *bifid* (hereafter *bi*) Gal4 driver (Calleja *et al.*, 1996; Lecuit *et al.*, 1996) to selectively target FLP on the dorso-ventral (DV) margins of the eye. The FLP will act on the DV margins to enable generation of genetic mosaic clones in a domain specific manner. Domain specific clonal analysis is essentially a useful strategy to maximize the yield of the clones of desired genotype and where sampling hundreds of clones are not required. There are several other eye specific enhancers which are being used like Glass Multiple Repeat (GMR)-FLP (Lee and Luo, 2001) or eyeless (ey)-FLP (Newsome *et al.*, 2000). But, these enhancers will not be able to address the requirement for the DV axis determination in the eye as GMR will drive expression in all the differentiating neurons and ey will drive expression in the entire early eye field.

We first investigated the expression of *bi*-Gal4 driver in the developing imaginal disc using a UAS-green fluorescent protein (GFP) reporter gene. In the early first instar eye imaginal disc, *bi*-Gal4 drives the expression of GFP (*bi>*GFP) reporter in a small subset of cells in the posterior margin in the glial precursor cells in the optic nerve (Fig. 1A). In the late first instar eye disc, *bi*-Gal4 expression refines and starts expressing in a couple of cells on the margin of the eye imaginal disc (Fig. 1B; arrow). In the early second instar, *bi*-Gal4 driven GFP reporter expression becomes robust in the cells on both the dorsal and ventral margins of the developing eye imaginal disc (Fig. 1C). During late second instar stage of development, the *bi*-Gal4 expression domain remain restricted to the dorsal and ventral eye margins and the number of cells expressing the GFP reporter is increased to 100–150 cells (Fig. 1D). In the third instar eye imaginal disc, *bi*-Gal4 expression is observed in the dorsal and ventral margins as well as in some glial cells within the eye field (Fig. 1E). The third instar eye imaginal discs exhibit clearly demarcated eye field as evident from the expression of the pan neural marker ELAV that marks the photoreceptor neurons (Fig. 1E).
Gal4 domain is restricted to the dorsal and ventral margins of the developing eye field right from early larval development. The adult eye pigmentation pattern which is dependent on expression of mini-white reporter exhibits DV specific distribution in bi-Gal4 flies. The mini-white expression is enriched on the dorsal and ventral margins of the adult eye and is absent near the equator (Fig. 1F).

The expression of bi-Gal4 driver is not only restricted to the developing eye imaginal disc but also present in the other developing fields including wing and leg imaginal discs (Lecuit et al., 1996). In the leg imaginal disc, bi-Gal4 can drive expression in a proximal subset of cells (Fig. 1G). In the wing imaginal disc, the expression of bi-Gal4 driven GFP reporter was observed in a domain which extends both in the anterior and posterior compartments as well as the dorsal and ventral margin, in the area which gives rise to the wing blade (Fig. 1H,I).

Using suitable genetic crosses to combine the FLP/FRT and Gal4/UAS system, we generated stocks where bi-Gal4 can drive expression of FLP within its expression domain, and trigger homologous recombination at the site of the FRT cassettes to generate homozygous loss-of-function clones (Fig. 2). We established fly stocks which allow generation of mutant clones for genetic mutations on particular arms (2R, 3R) of the second and third chromosomes. In the first set of flies, the genetic mosaic clones can be detected by loss of GFP (ubi-GFP) expression. Second, we tested the approach where the homozygous wild-type twin clones generated by recombination in somatic cells can be eliminated by using the “cell-lethal” mutations (Stowers and Schwarz, 1999; Newsome et al., 2000). The “cell-lethal” approach can prove advantageous to study the effects of loss-of-function of slow growing cells of mutant clones that are otherwise competed out by their wild-

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**FIG. 2.** Schematic of genetic crosses to generate reagents for the DV genetic mosaic system on the second and third chromosome.
type counterparts or neighbors. This approach can be useful in addressing the issues of cell-competition (Stowers and Schwarz, 1999; Newsome et al., 2000). Furthermore, these tools allow misexpression of transgenes (UAS-X) of interest in the dorsal and ventral margins of the developing eye. As misexpression of genes will be restricted to a smaller domain within the eye, it will facilitate the analysis of gene function which may otherwise be compromised by the death of the disc or organism.

**GENERATION OF CLONES**

We tested these stocks using the mutant alleles of *Lobe* (*L*), a gene involved in ventral eye development. Loss-of-function of *L* results in loss of the ventral eye whereas the dorsal clones do not have any effect (Singh and Choi, 2003). Earlier *L* clones were generated using an ey promoter which drives FLP expression in the entire developing eye field continuously (Reuter et al., 2003; Singh and Choi, 2003). Even though *L* is required for ventral eye development, it results in generation of *L* loss-of-function clones in the entire developing eye field during all stages of development. Loss-of-function clones of *L* using the bi-Gal4 driven FLP mediated recombination of FLP-recombinase target sequences (FRT) cassette on 2R resulted in selective loss of ventral eye (Fig. 3C,D). These clones were generated using the “cell-lethal” approach (Newsome et al., 2000). It has been shown that *L* loss-of-function phenotype can be rescued by blocking caspase dependent cell death (Singh et al., 2006) using misexpression of the baculovirus P35 transgene caspase inhibitor (Hay et al., 1994).

We also tested if our domain specific genetic mosaic approach can rescue this loss of ventral eye phenotype of *L* loss-of-function clones by blocking caspase dependent cell death. Misexpression of P35 on the dorsal and ventral margin using our bi-Gal4 driver can rescue the loss of ventral eye phenotype of *L* loss-of-function clones (Fig. 3E,F). We also tested the other strategy of making random clones by heat shock FLP or ey promoter linked to FLP to make clones of *L* both on dorsal and ventral margins of the developing eye and found similar phenotypes of loss of ventral eye (data not shown). This phenotype of loss of ventral in *L* mutant clones can be rescued by misexpression of P35 (Fig. 3E,F). The third chromosomal stocks were also used to generate clones of *bomotborax* (*btb*). As reported earlier, loss of function of *btb* resulted in enlargement of the ventral eye (Pai et al., 1998); we found similar ventral eye enlargements using our system (data not shown).

Thus, overall directed genetic mosaic approach using bi-Gal4 presents an efficient strategy to assay the effect of essential genes and their lethal mutations in specific tissues (domains) and time windows. Our results further suggest that these reagents will serve as an excellent tool for testing the function of genes involved in DV patterning of the eye, and genetic epistasis of genes involved in DV patterning of eye. Furthermore, these reagents can be used for genome-wide screening.
approaches using either loss-of-function clones or misexpression of a gene of interest only on the dorsal and ventral margins of the eye. The cells outside of the Gal4 expression domain in the eye imaginal disc may serve as the control. Temperature sensitive Gal80ts alleles are available which can block the Gal4 activity (McGuire et al., 2003, 2004). In the future, we can also introduce the Gal80ts in this bi-Gal4 mediated domain specific clone system to develop a more efficient temporal control. We anticipate this system will provide an efficient, genetic tool in the hands of fly researchers to study DV patterning in the Drosophila eye.

METHODS

Stocks used in this study were bi-Gal4 (Calleja et al., 1996; Lecuit et al., 1996); y, w; UAS-NLS-GFP<sup>S65T</sup> (Ito et al., 1997), UAS-FLP/TM6B Tb, UAS-FLP/CyO (Duffy et al., 1998), y, w; FRT42D cl w<sup>1</sup>/CyO (Newsome et al., 2000), y, w; FRT42D ubi-GFP/CyO, y, w; FRT82B ubi-GFP/TM6B Tb. These stocks are described in flybase (http://flybase.bio.indiana.edu). The flies were maintained on standard fly food at 25°C. An outline of the genetic crosses for generation of flies required for domain specific genetic mosaic approach is shown in Figure 2. The various genotypes used for this study are:

- bi-Gal4 (X); FRT42D ubi-GFP/CyO (II); UAS-FLP/TM6B Tb (III),
- bi-Gal4 (X); FRT42D cl w<sup>1</sup>/CyO (II); UAS-FLP/TM6B Tb (III),
- bi-Gal4 (X); UAS-FLP/CyO (II); FRT82 ubi-GFP/TM6B Tb (III).

Roman numerals denote the chromosomes. The UAS-FLP and FRT stocks were obtained from the Bloomington Stock Center.

Genetic Mosaic analysis

We used genetic mosaic approach to generate loss-of-function clones in the eye (Xu and Rubin, 1993). For the generation of clones in the eye, we have used bi-GAL4 driven UAS-FLP as source of FLP on the dorsal and ventral margin of the eye field. To generate mosaic clones of l in the eye, bi-GAL4; FRT42D ubi-GFP; UAS-FLP virgins were crossed to males of l<sup>rev</sup>FRT42D/CyO, (ii) l<sup>rev</sup>FRT42D/CyO; UAS-P35. To test third chromosomal reagent, we crossed bi-Gal4; UAS-FLP/CyO; FRT82 ubi-GFP/TM6B Tb virgins to y, w; FRT82B btb<sup>1003</sup>/TM6B Tb males. Mutant tissue was marked by the absence of GFP reporter in the eye imaginal disc. We also extended the bi-Gal4 mediated generation of loss-of-function clones where wild-type cells were eliminated using the cell lethal approach. All these reagents will be readily available to the research community upon acceptance of the manuscript.

Immunohistochemistry. Imaginal discs were dissected from first-, second-, and wandering third-instar larvae in 1XPBS and stained following the standard protocol (Singh et al., 2002). Antibodies used were rat anti-Elav (1:100), mouse anti-Wg (1:50) (Developmental Studies Hybridoma Bank), rabbit anti-Dlg (1:200; a gift from K. Cho). Secondary antibodies (Jackson Laboratories) used in this study were goat anti-rat IgG conjugated with Cy5 (1:200), donkey anti-rabbit IgG conjugated to Cy3 (1:250), donkey anti-rabbit IgG conjugated to FITC, and donkey anti-mouse IgG conjugated to Cy3 (1:200). Tissues were mounted on slides in Vectashield (Vector Laboratories). Immunofluorescent images were analyzed using the Olympus Fluoview 1000 Laser Scanning Confocal Microscope.

Bright field imaging. Adult eye images were taken on Axioimager.Z1 Zeiss Apotome. Adult flies were mounted on a needle and then individual image stacks were generated using Z-sectioning approach. The final image was generated using extended depth of focus function of Axiovision software version 4.6.3.

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LITERATURE CITED


A Glimpse Into Dorso-Ventral Patterning of the Drosophila Eye

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During organogenesis in all multi-cellular organisms, axial patterning is required to transform a single layer organ primordium into a three-dimensional organ. The Drosophila eye model serves as an excellent model to study axial patterning. Dorso-ventral (DV) axis determination is the first lineage restriction event during axial patterning of the Drosophila eye. The early Drosophila eye primordium has a default ventral fate, and the dorsal eye fate is established by onset of dorsal selector gene pannier (pnr) expression in a group of cells on the dorsal eye margin. The boundary between dorsal and ventral compartments called the equator is the site for Notch (N) activation, which triggers cell proliferation and differentiation. This review will focus on (1) chronology of events during DV axis determination; (2) how early division of eye into dorsal and ventral compartments contributes towards the growth and patterning of the fly retina, and (3) functions of DV patterning genes. Developmental Dynamics 241:69–84, 2012. © 2011 Wiley Periodicals, Inc.

Key words: Drosophila eye; eye development; axial patterning; dorso-ventral patterning; dorsal selector; ventral eye genes; Lobe; Serrate; Pannier; Homothorax; Teashirt; Wingless

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INTRODUCTION

During development, the patterning signals progressively restrict cell fates by subdividing a large developing field into smaller fields with limited developmental potential. These smaller fields that correspond to the domains of expression of selector genes are referred to as compartments. The selective spatio-temporal expression pattern of the cell fate selector genes is responsible for the formation of compartments (Blair, 2001; Curtiss et al., 2002; Held, 2002; Dahmann et al., 2011). The boundary between the compartments, where two different cell types are juxtaposed, is responsible for generating new signaling centers to regulate patterning, growth and differentiation of a developing field (Meinhardt, 1983; Blair, 2001). Thus, formation of the developmental boundaries is crucial for maintaining the downstream patterning events (Blair, 2001; Curtiss et al., 2002; Dahmann et al., 2011). Therefore, an important question in developmental biology is how these boundaries are generated and maintained during development. The aim of this review is to provide an overview of recent advances in our understanding of generation of the boundary between the dorsal and ventral compartment of the eye and its implications on development of the eye as an organ. This process is referred to as Dorso-Ventral (DV) patterning of the Drosophila eye. The Drosophila eye, an ideal model system for studying organogenesis, has been extensively used to investigate tissue patterning and cell–cell communication during axial patterning. Furthermore, Drosophila eye serves as an excellent model to understand the genetic mechanism responsible for division of a developing field into several smaller fields with positional fate restrictions (Singh et al., 2005b). The genetic machinery that controls Drosophila eye development closely resembles that of higher vertebrates, suggesting conservation of certain
genetic pathways throughout evolution (Wawersik and Maas, 2000; Gehring, 2005; Erdlik et al., 2009; Kumar, 2009). The axial pattern of the eye disc has not been well studied until recently due to the complexity of eye development.

Axial patterning, which is essential for organogenesis in all the multi-cellular organisms, involves formation of Antero-Posterior (AP), Dorso-Ventral (DV), and Proximo-Distal (PD) compartments (Cohen et al., 1993; Cohen, 1993). During axial patterning of the wing- and the leg-imaginal discs, the sequence of events involves the division of a field into anterior and posterior compartments of independent cell lineages, followed by subdivision of these imaginal discs into dorsal and ventral compartments. Interestingly, this sequence of division is not followed in the developing eye imaginal disc because it does not have analogous anterior-posterior axis. Instead, DV patterning is the first lineage restriction in the developing eye imaginal disc (Singh and Choi, 2003; Singh et al., 2005b). Despite the differences in the sequence of events, evidence suggests that some aspects of the DV patterning mechanism are highly conserved in the developing eye and the wing. An important common conclusion is that the border between DV compartments is a center for organizing the growth and patterning of the disc. In subsequent sections of the review, we will focus on the mechanism involved in generation of DV domains in the developing eye, and the genetic basis for the establishment of the DV pattern.

EYE IMAGINAL DISC

In Drosophila, a holometabolous insect, the primordia of all the adult structures are sequestered in the larva as epidermal invaginations that are called imaginal discs (Bodenstein, 1950; Ferris, 1950; Atkins and Mardon, 2009). The adult eye, antenna, head cuticle, and head structures develop from a common developing field called an eye-antennal imaginal disc (Cohen, 1993; Held, 2002). The regions of the eye-antennal imaginal disc, which give rise to head structures including pillarium, frons, and maxillary palpus, originate from five embryonic segments and the acron (Jurgena and Hartenstein, 1993; Younossi-Hartenstein and Hartenstein, 1993). The monolayer epithelium does not accurately reflect the sac-like anatomy of the imaginal discs (Gibson and Schubiger, 2001). Drosophila imaginal discs are a contiguous cell sheet of flattened epithelial cells with two opposing surfaces, a columnar epithelium called disc proper (DP) and a squamous peripodial epithelium called the peripodial membrane (PM) (McClure and Schubiger, 2005; Atkins and Mardon, 2009). The Drosophila retina develops from the DP while the PM of the eye-antennal disc gives rise to the adult head structures (Fig. 1; Milner et al., 1983; Hanyie and Bryant, 1986; Atkins and Mardon, 2009). The eye-antennal imaginal disc emerges from an embryonic imaginal primordium, which is an anterior-dorsal sac comprising approximately 20 cells that are set aside during mid-embryogenesis (Poulson, 1950; Garcia-Bellido and Merriam, 1969; Yamamoto, 1996). The embryonic precursors for imaginal discs grow asynchronously from the rest of the developing embryo (Anderson, 1972a,b; Crick and Lawrence, 1975; Cohen, 1993; Held, 2002; Kumar, 2011). In the first two larval stages, the eye imaginal disc cells divide and grow. The distinction between the developing antenna and the eye field begins to appear during the second instar larvae (Kumar and Moses, 2001; Kenyon et al., 2003; Dominguez and Casares, 2005; Atkins and Mardon, 2009). The developing eye field is further divided into precursors for the eye proper, head cuticle, and the ocelli while the antennal field divides into precursors for the antenna and head cuticle. Retinal differentiation begins from the posterior margin of the eye imaginal disc during late second instar or early third instar stage of larval development (Ready et al., 1976). Since the retinal differentiation is synchronous in nature, it appears like a wave of differentiation initiating at the posterior margin of eye imaginal disc, which then proceeds anteriorly. The wave of differentiation is referred to as the morphogenetic furrow (MF, Fig. 1 A, A', arrowhead). It results in transition of an undifferentiated epithelium to differentiated cell types comprised of regularly spaced photoreceptor clusters (Ready et al., 1976; Wolff and Ready, 1993). Posterior to the furrow, photoreceptor clusters are generated by a sequence of events including the selection of the R8 founder neuron and recruitments of additional photoreceptor precursors in the order of R2/5, R3/4, and R1/6/7 (Wolff and Ready, 1993; Kumar, 2011). Thus, if the eye imaginal disc is largely undifferentiated until second instar of development, an interesting issue is how compartments are identified in the Drosophila eye imaginal disc.

DORSAL AND VENTRAL COMPARTMENTS AND THE EQUATOR

The adult compound eye is comprised of approximately 800 unit eyes or ommatidia (Fig. 1D). Each ommatidium consists of eight photoreceptor neurons assembled in an asymmetric trapezoidal pattern, and when viewed from the top it resembles a honeycomb-like, hexagonal facet. The surrounding cell types are non-neuronal and include pigment, cone cells, and mechano-sensory bristles (Fig. 1E, F; Wolff and Ready, 1993). This pattern of organization is repeated in all ommatidia of the eye. However, despite the similarity in the cellular composition of each ommatidium, the spatial arrangement of ommatidia in the eye is organized in two orientations (Fig. 1E, F). These two orientations also serve as a marker to distinguish the dorsal and the ventral halves of adult eye. The photoreceptors are arranged in a trapezoidal fashion within an ommatidium. The ommatidial clusters within an eye are organized in a mirror asymmetry as they are polarized in the opposite directions (Fig. 1E, F). The boundary between the ommatidia of the dorsal half and their mirror image ventral ommatidia is referred to as an equator (Fig. 1E, F). This mirror symmetry, which corresponds to DV axis or compartments of the adult eye, has been described in many insect eyes (Dietrich, 1909). Since the developmental mechanisms underlying the DV pattern have not been studied in detail, it raises an interesting
question of how the dorsal and ventral pattern is established.

The pioneering studies to discern the relation between the equator and the DV compartmental boundary in the Drosophila eye suggested that the equator is not determined as the boundary between the dorsal and ventral cell lineages (Ready et al., 1976). Even though the result from this study does not exclude the possibility that the dorsal and the ventral domains of the eye derive from two independent cell lineages, the lineage boundary may not precisely correspond to the equator. A series of elegant genetic analysis experiments involving a large number of mosaic clones in the adult eye and the head supported this idea (Baker, 1978). These experiments demonstrated that clones strictly follow the DV boundary, and they do not intermingle near the DV border (Held, 2002). These results validated the hypothesis that Drosophila eye derives from DV compartments. The wing imaginal disc is divided into anterior and posterior groups of cells in its early stage of development, which is followed by further partitioning into dorsal and ventral compartments (Lawrence and Morata, 1976). To analyze whether the eye and the head are also subdivided into different domains by sequential compartmentalization as in the wing, another mosaic analysis was carried out. Nearly all clones (96%) were restricted to either dorsal or ventral domains of the eye, thereby confirming the presence of a boundary between the dorsal and ventral cells. Thus, clones generated in the dorsal or ventral compartment did not cross the DV boundary. A few clones (4%) did cross the DV border, which was probably due to the fact that such clones might have been induced prior to compartmentalization (formation of dorsal and ventral compartment boundary) or two independent dorsal and ventral clones might have juxtaposed at the equator region thereby giving a false notion of a single clone not respecting the DV boundary (Baker, 1978). The DV lineage restriction observed in the adult eye was also confirmed in the developing eye imaginal disc where large clones do not cross the DV midline and showed a sharp outline along the DV midline, and the clones located within the dorsal or ventral domain had wiggly borders (Domínguez and de Celis, 1998). Unlike the DV lineage restriction that is established in the first instar larval eye imaginal discs, there is evidence that anterior-posterior restriction occurs a little later in the second instar eye imaginal disc. Thus, the significance of this anterior-posterior restriction remains to be seen as the morphological distinction between the anterior and posterior regions. In subsequent sections, we discuss the role of DV patterning genes and the generation of pattern along the DV axis of the eye.

GENESIS OF THE EYE

The DV boundary has been suggested as the site for the activation of Notch (N) signaling in the eye imaginal disc to promote growth (de Celis et al., 1996; Go et al., 1998; Baonza and Garcia-Bellido, 2000). However, if DV patterning occurs as late as seen in the adult eye based on the orientation of the photoreceptors, then it may not be crucial for the growth of the eye. Thus, efforts were channeled towards investigating whether DV patterning takes place earlier during eye development. Seminal reports from three different groups established that DV lineage restriction takes place earlier during larval eye development due to domain-specific expression of the DV patterning genes (Cho and Choi, 1998; Domínguez and de Celis, 1998; Papayannopoulos et al., 1998). The DV patterning genes are a class of genes involved in generating and maintaining the DV lineage in the eye. These reports identified a new time line for the initiation of DV patterning to early larval development. They also identified the genes whose expression and/or function was restricted either to the dorsal or ventral compartment of the eye. Thus, it was proposed that DV patterning in the eye is generated by the domain-restricted expression of the dorsal and the ventral eye genes (Cho and Choi, 1998; Domínguez and de Celis, 1998; Papayannopoulos et al., 1998; Cho et al., 2000).

The basic question was what is the default state of the early eye primordium? To answer this question, several groups directed their efforts to identify the genes that are expressed in the early eye primordium. During embryonic development, the eye primordium begins as a homogenous group of cells that continue to grow during first larval instar to form the eye imaginal disc. All the cells of the first-instar eye imaginal disc uniformly express Lobe (L), a gene known to be involved in ventral eye development (Singh and Choi, 2003). These studies revealed that until the late first instar of larval eye development, the entire eye primordium is ventral in fate and depends on the function of ventral genes like L and its downstream target Serrate (Ser) (Singh and Choi, 2003; Singh et al., 2005b; Kumar, 2011). Loss-of-function of the L gene, which is expressed ubiquitously in the eye imaginal disc (see Fig. 3A), results in selective growth defects in the ventral half of the eye (Figs. 2, 3C, D). The loss-of-function studies suggested that the requirement of L function evolves along the temporal axis (Singh and Choi, 2003; Singh et al., 2005b). During early eye development, loss-of-function of L results in the complete loss of eye field (Fig. 3B). However, loss of L gene function later during eye development causes selective loss of the ventral half of the eye (Singh et al., 2005b). Loss-of-function of Ser also results in the similar loss of the ventral eye phenotype (Table 1; Singh and Choi, 2003; Singh et al., 2005b). Interestingly, the timing of restriction of L/Ser functional domain from the entire developing eye field (Fig. 3D, E) to only the ventral half of the eye (Fig. 3B, C) corresponds to the onset of pannier (pnr) gene expression along the dorsal margin of the eye (Table 1; Fig. 2). During late first instar of eye development, the entire homogenous population of the ventral cells of the eye primordium transition into two distinct dorsal and ventral lineages with the onset of pnr expression on the dorsal eye margin (Singh and Choi, 2003). This suggests that the ventral fate is the ground state of the larval eye imaginal disc, and L and Ser are essential for survival and/or maintenance of this ventral state (Singh and Choi, 2003; Singh et al., 2005b, 2006).
Fig. 1.

Fig. 2.
**DV Patterning in the Eye**

**Genes Regulating Ventral Eye Growth**

*L*, a gene involved in ventral eye development (Table 1), was first reported in 1925, as a gene required for eye growth (Morgan et al., 1925) and was cloned in 2002 (Chern and Choi, 2002). *L* encodes an ortholog of PRAS40 (Oshiro et al., 2007; Vander Haar et al., 2007; Wang and Huang, 2009), and it is required during all stages of larval eye development (Chern and Choi, 2002; Singh et al., 2005b). *L* protein is expressed in both dorsal and ventral domains throughout eye imaginal disc development (Fig. 3A). Even though *L* is expressed uniformly in the entire eye field, *L* function is not required for growth and differentiation in the dorsal region of the eye (Chern and Choi, 2002; Singh et al., 2005b). Evidence for this “domain-specific” function of *L* (ventral specificity) came from genetic mosaic analysis using a null mutation (Chern and Choi, 2002; Singh and Choi, 2003). Loss-of-function clones of *L* show an eye suppression phenotype specifically in the ventral eye; however, the dorsal clones do not suppress eye fate and exhibit well-organized photoreceptor clusters (Chern and Choi, 2002; Singh and Choi, 2003). Further genetic analysis revealed that this domain-specific *L* function in growth was downstream to N-signaling, mediating N function either in the same or parallel pathway (Chern and Choi, 2002). Thus, the growth of early eye disc is controlled asymmetrically in the dorsal and ventral domains. Expression of *L* in both dorsal and ventral domains is puzzling since its function is only required for growth of the ventral domain. It is possible that an unidentified partner that is expressed in the dorsal domain of the eye imaginal disc antagonizes the *L* function, or *L* may be selectively activated by some yet to be identified partner in the ventral domain.

Another candidate gene that may be contributing to ventral eye development is *decapentaplegic* (*dpp*), a member of the TGF-β family of proteins, which acts as a long-range secreted morphogen (Table 1; Nellen et al., 1996; Chanut and Heberlein, 1997). *Dpp* forms a gradient in the early eye anlage (anterior brain and eye field) that transverses from dorsal to ventral (Chang et al., 2001). In the early eye imaginal disc, *Dpp* is preferentially expressed in the ventral eye domain (Cho et al., 2000). In *dpp* mutants, the ventral part of early eye disc exhibits similar pattern defects as seen in *L* mutants. This *dpp* phenotype may be an outcome of ectopic induction of *prn* or *wg* expression in the ventral domain as observed in *L* mutants (Singh et al., 2005a). In the DP of early eye imaginal disc *Dpp*, Hedgehog (*Hh*) and Wg signaling from the peripodial membrane is required to trigger N activation. Similar to limb patterning and development (Brook and Cohen, 1996; Penton and Hoffmann, 1996; Theisen et al., 1996), during eye imaginal disc development, *Dpp* antagonizes Wg. This antagonistic interaction occurs in the peripodial membrane across the DV border (Cho et al., 2000). Thus, *Dpp* signaling plays a role in inducing DV polarity from peripodial membrane.

Ser is the N ligand in the ventral domain of the eye imaginal disc (Table 1; Speicher et al., 1994; Cho and Choi, 1998; Domínguez and de Celis, 1998; Papayannopoulos et al., 1998; Domínguez and Casares, 2005). *Ser* acts downstream of *L* in the ventral eye as *Ser* transcription is repressed in early eye discs from *L* homozygous larvae. Evidence for this conclusion comes from the *Ser-lacZ* reporter expression. Interestingly, *Ser-lacZ* expression in the posterior medial region remains at a significant level. Loss-of-function clones of *L* cause strong reduction of *Ser* in the ventral eye whereas increased levels of *L* using the “flip out” approach induce *Ser* expression even in the dorsal domain of eye imaginal disc. Thus, *L* that acts downstream to *N* may be involved in inducing the *Ser* expression in the developing eye imaginal disc (Chern and Choi, 2002). Hypomorphic alleles of *Ser* exhibit reduced eye size suggesting a role for *Ser* in eye development. However, *Ser* loss-of-function clones do not exhibit any phenotypes in the eye (Sun and Artavanis-Tsakonas, 1996; Papayannopoulos et al., 1998; Chern and Choi, 2002), but mis-expression of the dominant-negative form of *Ser* (*SerDN*) causes severe growth defects in the eye imaginal disc (Kumar and Moses, 2001; Singh and Choi, 2003). It is possible that *Ser* function may be compensated by another factor, or *Ser* may somehow be secreted or transcytosed into...
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<th><strong>Drosophila</strong></th>
<th><strong>Vertebrate homolog</strong></th>
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<tr>
<td>fringe (fr)</td>
<td>Lunatic fringe</td>
<td>Glycosyl transferase</td>
<td>Secreted signaling protein, DV boundary formation</td>
<td>Irvine and Wieschaus, 1994; Cho and Choi, 1998; Dominguez and de Celis, 1998; Papayannopoulos et al., 1998</td>
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<td>Serrate (Ser)</td>
<td>Jagged-1</td>
<td>Ventral N ligand</td>
<td>Ventral eye growth and development</td>
<td>Speicher et al., 1994; Cho and Choi, 1998; Dominguez and de Celis, 1998; Papayannopoulos et al., 1998; Cho et al., 2000</td>
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<td>Chip</td>
<td>Nl/Ldb1/Clim-2</td>
<td>Transcription co-factor</td>
<td>Define ventral eye boundary</td>
<td>Roiinant et al., 2010</td>
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<td>Sloppy paired (Slp)</td>
<td>BF-1 (not complete homology)</td>
<td>Forkhead transcription factor</td>
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<td>decapentaplegic (dpp)</td>
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<td>pannier (pnr)</td>
<td>GATA-1</td>
<td>Zinc finger, GATA family</td>
<td>Dorsal eye fate selector</td>
<td>Ramain et al., 1993; Maurel-Zaffran and Treisman, 2000; Gomez-Skarmeta and Modolell, 2002; Singh et al., 2005b; Oros et al., 2010</td>
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<td>araucan (ara)</td>
<td>Irx 1, 3</td>
<td>Homeodomain</td>
<td>Dorsal eye fate selector</td>
<td>Gomez-Skarmeta and Modolell, 1996; Cavodeassi et al., 1999; Pichaud and Casares, 2000; Gomez-Skarmeta and Modolell, 2002</td>
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<td>mirror (mirr)</td>
<td>Irx 4, 6</td>
<td>Homeodomain</td>
<td>Dorsal eye fate selector</td>
<td>McNeil et al., 1997; Heberlein et al., 1998; Kehl et al., 1998; Yang et al., 1999; Singh et al., 2005b</td>
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<td>Delta (Dl)</td>
<td>Delta like 3 (DLL3)</td>
<td>Transmembrane Notch Ligand</td>
<td>Dorsal Notch (N) Ligand</td>
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<td>homothorax (hth)</td>
<td>Meis</td>
<td>Homeodomain</td>
<td>Ventral eye suppression</td>
<td>Rieckhof et al., 1997; Pai et al., 1998; Ryoo et al., 1999; Pichaud and Casares, 2000; Dominguez and Casares, 2005; Singh et al., 2005b; Bessa et al., 2008</td>
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<td>teashirt (tsh)</td>
<td>TSH1, TSH2, TSH3</td>
<td>C2H2 zinc finger transcription factor</td>
<td>Dorsal eye growth, ventral eye suppression</td>
<td>Fasano et al., 1991; Pan and Rubin, 1998; Bessa et al., 2002; Singh et al., 2002, 2004; Datta et al., 2008</td>
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<td>Lobe (L)</td>
<td>PRAS40 (Ortholog)</td>
<td>Proline rich Akt substrate</td>
<td>Ventral eye growth, no affect on dorsal eye</td>
<td>Chern and Choi, 2002; Singh and Choi, 2003; Singh et al., 2005a,b, 2006; Wang and Huang, 2009</td>
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<tr>
<td>Expression on both margins</td>
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<td>optomotor blind (omb)</td>
<td>Tbx5</td>
<td>Transcription factor</td>
<td>Expressed on dorsal and ventral eye margin. Not known.</td>
<td>Calleja et al., 1996; Singh et al., 2004</td>
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neighboring cells as shown in experiments performed using a cell culture system (Klueg and Muskavitch, 1999; Kumar and Moses, 2001; Singh et al., 2005b). This may explain the apparent lack of phenotype in Ser mutant clones. Misexpression of Ser \text{DN} in early eye imaginal disc using 	ext{ey}-	ext{Gal4} (Hazelett et al., 1998) results in either preferential loss of ventral eye or loss of the entire eye (Singh and Choi, 2003). Misexpression of Ser \text{DN} in random gain-of-function clones generated by the “flip-out” method (Pignoni and Zipursky, 1997) results in suppression of eye fate in the ventral half of the eye. The similar phenotypes of Ser \text{DN} misexpression and L mutants in the eye disc further validate that L and Ser work in the same pathway to regulate the growth of the ventral eye domain.

It is known that Ser can activate N only at the DV border since Ser-N interaction is prevented by Fringe (Fng) in the ventral domain cells away from the DV border of the eye imaginal disc. Fng is a glucosaminyltransferase that elongates O-linked fucose residues to the EGF domains of N (Okajima and Irvine, 2002). Fng is known to bind N to promote N-Delta (Dl) interaction and is required to restrict N activation at the DV border (Irvine and Wieschaus, 1994; Kim et al., 1995; Fleming et al., 1997). Contrary to the positive function of Fng in N-Dl interaction, Fng inhibits Ser-N interaction when it is bound to N protein (Ju et al., 2000; Singh et al., 2005b). As a result, the N activation by Dl is enhanced only at the DV border. The expression pattern of these DV-patterning genes changes dynamically in the developing eye imaginal disc, thereby showing striking differences before and after the initiation of retinal differentiation. Initially, \text{fng} is expressed in the ventral domain of the eye imaginal disc, but as the eye imaginal disc undergoes retinal differentiation and the morphogenetic furrow proceeds anteriorly, \text{fng} expression further evolves. At this stage, \text{fng} exhibits preferential localization anterior to the furrow in both the dorsal and ventral eye domain. These results validate the conclusion of genetic mosaic studies, which suggested that DV pattern is established during early eye development prior to retinal differentiation. The essential role of Fng in DV patterning was demonstrated by analysis of \text{fng} mutant clones. In loss-of-function clones of \text{fng} in the ventral eye, DV polarity is reorganized near the ectopic \text{fng}+/\text{fng}− border resulting in non-autonomous polarity reversals. This leads to the generation of de novo equators and ectopic localized activation of N at the \text{fng}+/\text{fng}− boundary (de Celis et al., 1996; Cho and Choi, 1998; Go et al., 1998; Baonza and Garcia-Bellido, 2000).

The DV axis in the wing imaginal disc is inverted in comparison to the eye imaginal disc. In the eye imaginal disc, Dl and Ser are preferentially expressed in the dorsal and ventral domains, respectively. However, the localization of Dl and Ser preferential expression is reversed in the developing wing imaginal disc. The inversion of the DV axis in the eye and the wing disc may be due to the fact that the eye disc rotates 180° during embryogenesis (Struhl, 1981). Therefore, Ser functions as an N ligand in the dorsal cells, whereas Dl is the N ligand in the ventral cells. Not surprisingly, \text{fng} is ventral-specific in the eye but dorsal-specific in the wing imaginal disc.

Other candidate genes involved in ventral eye development are \text{Chip} and \text{sloppy paired (slp)} (Table 1). \text{Chip}, a transcriptional co-factor, is required for ventral eye development (Rogniant et al., 2010). \text{Chip}, a ubiquitous transcriptional co-factor, interacts with classes of transcription factors during neural development. Chip has been reported to establish the ventral boundary of the eye and the head tissue (Rogniant et al., 2010). \text{Slp} belongs to the forkhead family of transcription factors, which are required for embryonic patterning (Grossniklaus et al., 1992). \text{Slp} locus has two transcription units. Both of them are expressed in the ventral eye and are functionally redundant (Sato and Tomlinson, 2007). \text{Slp} and Iro-C proteins have been shown to repress each other at the DV midline. N signaling at the DV midline suppresses \text{Slp} at the midline (Sato and Tomlinson, 2007).

**Dorsal Selector Genes**

It is known that compartment boundaries are defined by the spatio-temporal expression of genes (Blair, 2001; Curtiss et al., 2002; Dahmann et al., 2011). For example, \text{engrailed (en)} and \text{apterous (ap)} are expressed in the posterior and the dorsal compartments of the wing imaginal disc, respectively (Brower, 1988; Cohen et al., 1992; Hidalgo, 1998; Held, 2002). Thus, “Selector” genes were identified that assign a unique property to the cells within their expression domains, which results in the formation of unique territories (Blair, 2001; Curtiss et al., 2002). Moreover, loss-of-function of these selector genes results in the loss of that particular fate. In the Drosophila eye, the presence of these selector genes (which exhibit dorsal or ventral domain specific expression) became apparent in the earlier enhancer trap screens (Bier et al., 1989; Bhojwani et al., 1995; Sun et al., 1995). Enhancer trap lines containing \text{mini-white (w)} and \text{lacZ} reporter gene (P-lacW) (Bellen et al., 1989; Bier et al., 1989; Wilson et al., 1989; Bhojwani et al., 1995; Sun et al., 1995) that show domain-specific expression in the eye were isolated in the screens. Interestingly, some of these lines have \text{w}+ expression restricted only to the dorsal half of the adult eye. These enhancer trap lines have made significant contributions towards understanding the DV patterning in the eye (Choi et al., 1996; Sun and Artavanis-Tsakonas, 1996; McNeill et al., 1997; Kehl et al., 1998; Morrison and Halder, 2010). Most of these dorsal-specific P insertion lines were mapped to the chromosomal region 69CD, identifying this region as a hot spot for P-lacW insertions that show dorsal eye-specific expression. The molecular characterization of this 69CD chromosomal region revealed the existence of a cluster of homeobox genes, \text{arauca}n (\text{arauc}), \text{cau}polican (\text{caup}), and \text{mirror (mirr)} (Gomez-Skarmeta and Modolell, 1996; McNeill et al., 1997; Grillenzi et al., 1998; Heberlein et al., 1998; Kehl et al., 1998; Singh et al., 2005b). This cluster of homeobox genes that are located within an approximately 140-Kb region (Netter et al., 1998) are expressed in the dorsal half of the eye. They are referred to as Iroquois complex (Iro-C) because the mutation in these genes lacks lateral thoracic bristles and resembles the hair style.
Fig. 3. Lobe (L) and Serrate (Ser) are required for cell survival in the developing eye imaginal disc. A: In the wild-type eye imaginal disc, L (green) expression is ubiquitous. Elav (red) marks the photoreceptor neurons. B: Wild-type adult eye. C, D: Loss of L results in the preferential loss of ventral half of the (C) developing eye imaginal disc, and (D) the adult eye. C: Eye imaginal discs stained for Wg (green) to identify dorsal versus ventral eye imaginal disc compartment. The boundary of the eye field is as outlined in C (white) and D (black) showing preferential loss of the ventral eye. E, F: Early loss-of-function of Ser by misexpressing dominant-negative form of Ser in the entire eye imaginal disc (Kumar and Moses, 2001; Singh and Choi, 2003) using an ey-Gal4 driver (Hazelett et al., 1998; Singh and Choi, 2003) results in complete loss of the eye field both in (E) the eye imaginal disc and (F) the adult.

Fig. 4. Developmental Dynamics
of the Indian tribe, the Iroquois, also referred to as Mohawks (a native tribe that shaved all but a medial stripe of hairs on the head) (Gomez-Skarmeta and Modolell, 1996; Leys et al., 1996). They named the genes Araucan and Caupolican in honor of Amerindian tribes: Aracaunians and one of their heroes Caupolican.

The members of Iro-C are highly conserved, essential genes and exhibit significant differences in their expression pattern (Gomez-Skarmeta and Modolell, 2002). Mirr is strongly and dynamically expressed in the CNS (Netter et al., 1998; Urbach and Technau, 2003) and it is essential for follicle cell patterning (Jordan et al., 2000) while Ara and Caup are preferentially expressed in mesodermal tissues in the embryos (Netter et al., 1998). However, in the eye imaginal disc, all three Iro-C members are expressed in the dorsal half (Fig. 4E), raising a possibility that they might be functionally redundant. Loss-of-function of the mirr^{448} allele shows weak but significant defects of non-autonomous DV polarity reversals in comparison to mirr^{+} ommatidia in the dorsal half of the eye (McNeill et al., 1997). Compartments of different cell lineages do not intermingle due to differences in cell identities and affinities (Garcia-Bellido et al., 1973; Irvine, 1999; Dahmann et al., 2011). Somatic clones of cells lacking mirr function in the dorsal half of the eye exhibit smooth clone borders, indicating that cells lacking mirr avoid mixing with the neighboring mirr-expressing cells. However, the clones in the ventral half where mirr is not expressed show wiggly clone borders (Yang et al., 1999). This analysis suggests that mirr functions as a dorsal fate selector. Since the phenotype of mirr clones was not strong enough, it raised the possibility that ara and caup, the other two members of Iro-C, can partly compensate for the loss of mirr function in the eye. The issue of functional redundancy got resolved when a deficiency iro^{DMF3} was uncovered on three Iro-C genes by the deletion of ara and caup as well as a 5'-region of mirr (Gomez-Skarmeta et al., 1996; Diez del Corral et al., 1999), was employed for clonal analysis. Loss-of-function clones of iro^{DMF3} in the eye showed repolarization of the ommatidial polarity in the dorsal clones along with dorsal eye enlargement or formation of an ectopic eye field on the dorsal margin. There was no phenotype in the ventral half of the eye (Fig. 4F, G). These results further highlighted the importance of a boundary between the dorsal and ventral cell types. These results strongly support that the three Iro-C genes are partially redundant, and the Iro-C as a whole is required for organizing the DV polarity pattern and growth of the eye.

Loss-of-function of iro^{DMF3} also suggested that Iro-C genes function as dorsal selectors for head structures as well since mutant clones in the dorsal region induce the formation of ventral head structures (Cavodeassi et al., 2000). Ectopic ventral head tissues that resulted from loss of Iro-C genes are cell-autonomous and, therefore, accompanied by loss of corresponding dorsal structures. In contrast, ectopic ventral eyes are generated non-cell-autonomously since reversals of DV ommatidial polarity are detected in Iro-C^{+} wild-type region adjacent to the mutant clones. This also supports the idea that the DV boundary is an organizing center for DV pattern and growth in the eye imaginal disc. Furthermore, DV patterning of the eye occurs in earlier larval stages than the head patterning. In the Drosophila eye, pnr, another dorsal gene, which is expressed in the dorsal eye margin (Fig. 4A), exhibits similar loss-of-function (Table 1; Fig. 4B, C) and gain-of-function (Fig. 4D) phenotypes as observed with Iro-C in the eye and the head (Maurel-Zaffran and Treisman, 2000; Pichaud and Casares, 2000; Singh et al., 2005b; Oros et al., 2010). Pnr, a GATA-1 transcription factor, plays an important role in dorsal eye development, and acts as a selector for dorsal eye fate (Ramain et al., 1993; Maurel-Zaffran and Treisman, 2000; Pichaud and Casares, 2000; Dominguez and Casares, 2005; Singh et al., 2005b; Oros et al., 2010). In the hierarchy of dorsal genes, pnr is the top-most gene, and it induces Wingless (Wg), which, in turn, induces the expression of downstream target genes mirr in the dorsal half of the eye (Maurel-Zaffran and Treisman, 2000; Dominguez and Casares, 2005; Singh et al., 2005b). During later stages of development, which correspond to the retinal differentiation stage in late second instar and third instar of larval eye development, pnr is involved in defining the dorsal eye margin by regulating the retinal determination (RD) genes (Oros et al., 2010).

Wg, a secretory protein and a morphogen, is expressed along the antero-lateral margins of the third-instar eye imaginal disc (Table 1; Baker, 1988). Wg plays multiple roles during eye development. One of these roles of Wg is to promote growth of the early eye imaginal disc. During early eye development, Wg expression is restricted to the dorsal eye domain (Cho et al., 2000; Chang et al., 2001). During retinal differentiation stage, Wg is known to prevent ectopic induction of retinal differentiation from the lateral eye imaginal disc margin (Ma and Moses, 1995; Treisman and Rubin, 1995). Thus, Wg that acts as a negative regulator of the eye during retinal differentiation functions as a dorsal eye fate gene. Dl, an N ligand in the dorsal eye imaginal disc, has been assigned to the dorsal gene category in the early eye imaginal disc.

**Fig. 4.** Pnr and Iro-C members function as dorsal eye fate selectors. A: Pnr expression (green) is restricted to the dorsal eye margin of the developing eye imaginal disc (Maurel-Zaffran and Treisman, 2000; Pichaud and Casares, 2000; Singh and Choi, 2003). Elav (red) marks the photoreceptor neurons. B, C: Loss-of-function clones of pnr result in the enlargement of the existing dorsal eye field (e.g., in the clone outlined in B) in the eye imaginal disc (B) and adult eye (C). B: Note that there is a non-autonomous eye enlargement in the eye imaginal disc, which is attributed to generation of a de novo equator in the dorsal compartment of the eye imaginal disc (Maurel-Zaffran and Treisman, 2000; Oros et al., 2010). D: Misexpression of pnr (ey>GFP) in the eye imaginal disc suppresses eye fate, validating a late function of pnr in defining the eye field boundary (Oros et al., 2010). E: The expression domain of the members of Iroquois complex (Iro-C::GFP, green) spans the dorsal region of the eye imaginal disc. F, G: Loss-of-function of Iro-C causes dorsal eye enlargements in the (F) eye imaginal disc and in (G) adult eye. These phenotypes are similar to the (B, C) pnr loss-of-function phenotypes. H: Misexpression of ara, a member of Iro-C, in the entire eye imaginal disc (ey>ara) results in small eye. D, Dorsal; V, ventral.
GENES WITH DOMAIN SPECIFIC GROWTH RESPONSE: ROLE OF TEASHIRT (TSH) AND HOMOTHORAX (HTH)

In addition to the genes that exhibit DV domain-specific expression during patterning, there is a group of genes, which show differential functions in the dorsal-ventral compartments, but are not expressed in a DV-specific pattern. These genes can be broadly classified into two groups: (1) Genes expressed uniformly in the eye imaginal disc but their functional domain is restricted only to the ventral half of the eye, for example L and hth (Table 1); (2) Genes that are expressed uniformly in the early eye imaginal disc function differently in the dorsal and ventral half of the eye, for example, tsh (Table 1).

1. Hth: Hth a vertebrate homolog of murine proto-oncogene MEIS1 (Moskow et al., 1995), encodes a homeodomain transcription factor of the three-amino-acid extension loop (TALE) sub-family (Rieckhof et al., 1997). Like L, hth is expressed in the entire early eye primordium. However, with the onset of differentiation in the eye, Hth expression gets restricted to the cells anterior to the furrow (Fig. 5A, A'; Pai et al., 1998; Pichaud and Casares, 2000; Bessa et al., 2002; Singh et al., 2002a). Even though hth is expressed anterior to the furrow both in the dorsal and ventral half of the eye imaginal disc (Fig. 5A, A'), the loss-of-function of hth causes eye enlargement only in the ventral eye margin (Fig. 5B, B'; Pai et al., 1998; Bessa et al., 2002; Singh et al., 2002a). ELAV (red), a pan neural marker, marks the photoreceptors neurons in the eye imaginal disc. B, B': Loss-of-function clones of hth marked by the absence of the GFP reporter (clonal boundary marked by white dotted line) in the ventral eye results in eye enlargement, whereas in the dorsal eye these clones do not have any effect. C, D: Misexpression of hth in the entire eye using ey-Gal4 driver (ey->hth) results in a reduced eye field as seen in (C) the eye imaginal disc and (D) adult eye (Pai et al., 1998).
MH domain (for Meis and Hth), and a C-terminal region including the homeodomain (HD) (Rieckhof et al., 1997; Kurant et al., 1998; Pai et al., 1998; Noro et al., 2006). Alternative splicing is known to provide additional complexity to the genes encoding the Hth transcription factors (Glazov et al., 2005; Noro et al., 2006). Hth forms a heterodimer with Exd through its MH domain and translocates into the nucleus to regulate transcription (Ryoo et al., 1999; Jaw et al., 2000; Stevens and Mann, 2007). Since Exd is present in the entire eye, the ventral specific function of Hth has been proposed through its interaction with Wg and Tsh. Together they are involved in suppression of eye fate on the ventral margin.

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(Fasano et al., 1991). Tsh plays an important role during Drosophila eye development (Pan and Rubin, 1998; Bessa et al., 2002; Singh et al., 2002a, 2005b; Datta et al., 2009; Kumar, 2009, 2010, 2011). tsh is expressed both in dorsal and ventral eye anterior to the furrow, and it exhibits a DV constraint in its function. In the ventral eye, tsh acts as a repressor of eye fate, whereas in the dorsal eye, it promotes eye development (Fig. 6; Singh et al., 2002b, 2004). Interestingly, the DV constraint in tsh function in the eye stems from the partners with which it collaborates in the dorsal or the ventral eye disc (Fig. 6; Singh et al., 2004). It was shown that Tsh cooperates with Iro-Complex members and Dl in the dorsal eye for its growth promotion function (Fig. 6; Singh et al., 2004). The function of tsh in the ventral eye is dependent on Hth and Ser. The expression of tsh overlaps with hth in the eye imaginal disc, and like hth, tsh expression also evolves during larval eye development. Initially, in the first-instar eye imaginal disc, tsh is expressed in the entire eye imaginal disc but its expression retracts anteriorly to nearly three-quarters of the eye imaginal disc when the retinal differentiation begins (Bessa et al., 2002; Singh et al., 2002a). Furthermore, Tsh and Hth physically interact with each other [along with Pax-6 homolog, Eyeless (Ey)] to repress the expression of downstream target genes (Bessa et al., 2002; Dominguez and Casares, 2005). Further insights into the potential mechanism of tsh and hth in regulating growth and differentiation in the eye came initially from analysis of expression patterns of the retinal determination (RD) gene network members (Bessa et al., 2002). It has been proposed that Tsh, Hth, and Ey coexpress in the proliferating cells anterior to furrow to block precocious retinal differentiation and promote cell proliferation (Bessa et al., 2002; Singh et al., 2002a; Dominguez and Casares, 2005). All these studies suggest that DV patterning genes contribute towards the growth of the eye field.

BOUNDARY FORMATION DURING ORGANOGENESIS

One of the important questions is how organ size and growth are regulated by DV patterning genes in the eye. The dorsal selector genes assign a dorsal fate, and, thereby, generate a group of cells with unique properties that make them different from the default ventral state cells of the developing eye disc. Interestingly, the boundary between the dorsal and ventral cells is maintained by the antagonistic interactions between the genes required for the growth and development of the dorsal and ventral domains of the eye (Fig. 7; Singh et al., 2005a). It has been shown that L is essential for growth of the ventral eye tissue, but it is dispensable in the dorsal region specified by pnr function (Singh and Choi, 2003). In addition to a boundary between the dorsal and ventral compartment within the eye, a boundary is defined between the developing eye field and the surrounding head cuticle on the dorsal and ventral margins (Fig. 7). Since the adult eye, head cuticle, and other mouthparts are generated from the eye-antennal imaginal disc, there is a sequential fate restriction between the developing eye and head cuticle. Interestingly, these DV-patterning genes play an important role in defining the boundary of the eye field on the dorsal and the ventral margins (Oros et al., 2010). The boundary between the eye field and the head cuticle on the dorsal margin is regulated by pnr (Fig. 7). It has been shown that pnr function evolves during eye development. During early second instar of development, pnr is required for defining the dorsal lineage, before the onset of retinal differentiation, by inducing Wg and members of the Iro-C complex (Maurel-Zafran and Treisman, 2000; Singh and Choi, 2003; Singh et al., 2005b; Oros et al., 2010). However, later during the late second instar stage of eye development, when the morphogenetic furrow (MF) is initiated, pnr suppresses the photoreceptor differentiation at the dorsal eye margin (Oros et al., 2010). The endogenous expression of pnr is only in the peripodial membrane of the dorsal eye margin, which gives rise to the adult head cuticle. Loss-of-function clones of pnr exhibit ectopic dorsal eyes, which are restricted within the clones, and suggests that absence of pnr function promotes ectopic eye formation in the dorsal eye margin. Thus, Pnr defines the boundary between the head cuticle and the dorsal margin of the developing eye field (Fig. 7; Oros et al., 2010). Since pnr is not expressed in the ventral eye, there is a different mechanism to define the boundary of the eye field on the ventral margin. The boundary of the eye field on the ventral eye margin is defined by the antagonistic interaction of L with hth (Singh et al., 2011). In the ventral eye, transcriptional co-factor Chip interacts with the LIM-homeodomain proteins to define the boundary of the eye field (Roignant and Treisman, 2009). Interestingly, Chip-mediated regulation of the ventral eye boundary is independent of hth (Roignant and Treisman, 2009). Thus, the genetic cascade that regulates the boundary of the eye field on the dorsal and the ventral margin of the eye is different.

CONCLUDING REMARKS

Our understanding of the axial patterning of the Drosophila eye is far from complete. In this review, we have described the overview of key developmental events and genes involved in early DV patterning. The DV compartment formation is a key to initiate patterning and growth in the early eye imaginal disc. The present information clearly illustrates that DV patterning is required to initiate the generation of heterogeneous population (dorsal and ventral cell fate) of cells within a homogenous (default ventral fate) early eye primordial. Although our knowledge on the DV patterning in the eye has dramatically increased in recent years, we still do not know the molecular interactions important for the regulation of DV patterning. Moreover, many more genes (both known and novel) are expected to be involved in DV patterning, and future studies using novel genetic and
bioinformatics approaches should help in defining the full complement of genes involved in this intricate process. Identification and functional analysis of more molecular players involved in this process will help provide a better picture of how a small number of cells in the disc primordium grow to form a precise pattern of mirror symmetry in the compound eye. Furthermore, the possibility of crosstalk of the DV patterning pathway with other signaling pathways to regulate growth during the early phase of eye development cannot be ruled out. All this information will lay a foundation about understanding the process of organogenesis, as loss-of-function of the genes involved in DV patterning results in loss of the eye field or a part of the eye field. The complexity and precision of the neural connectivity in the adult visual system has fascinated researchers for a long time. The DV polarity of the retina is responsible for controlling the targeting of the retinal axon projections to the brain in humans and other higher vertebrates. Thus, DV patterning genes also contribute towards the wiring of the brain to the retina. How all these different facets work together to define the final form of this complex eye structure is an open question and is of fundamental importance.

SIMILARITIES WITH VERTEBRATE EYE

The basic sensory epithelium design of the vertebrate and most invertebrate eyes including *Drosophila* eye is similar (Charlton-Perkins and Cook, 2010; Sanes and Zipursky, 2010). The morphogenetic furrow (MF) in the fly eye is analogous to the wave of neurogenesis in the vertebrate retina (Neumann and Nusslein-Volhard, 2000; Hartenstein and Reh, 2002). Recent studies in the vertebrate visual systems have identified several genes that are expressed in a DV domain-specific manner in the retina. BMP-4 and Tbx5, act to restrict the expression of Vax2 and Pax2 to the ventral domain of the eye (Koshiba-Takeuchi et al., 2000; Mui et al., 2002; Peters, 2002; Peters and Cepko, 2002). These DV expression domains correspond to the developmental compartments (Peters, 2002; Peters and Cepko, 2002). The DV patterning plays an important role in retinotectal projection pattern (Koshiba-Takeuchi et al., 2000; McLaughlin et al., 2003). The R-cell projections form a precise topographic connection with the optic lobe, and are referred to as retinotopy, which is common to both the vertebrate and the insect visual system (Gaul, 2002). Furthermore, Jagged-1 (Jag1), a vertebrate homolog of the *Drosophila* ventral eye gene Ser, shows a DV asymmetric expression pattern in the retina. Moreover, the loss-of-function of Jag1 results in Alagille's syndrome, which also affects the eye (Oda et al., 1997; Xue et al., 1999; Kim and Fulton, 2007). Interestingly, it has been shown that mouse retina also begins with a default ventral-like state (Murali et al., 2005). Therefore, the DV boundary may play conserved roles in organizing growth and pattern of visual system in higher animals, and studies in *Drosophila* will further our knowledge in the area of animal development mechanisms and help to unravel the genetic underpinnings of developmental defects caused by mutations in human homologs of *Drosophila* DV patterning genes.

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Opposing interactions between homothorax and Lobe define the ventral eye margin of Drosophila eye

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ABSTRACT

Patterning in multi-cellular organisms involves progressive restriction of cell fates by generation of boundaries to divide an organ primordium into smaller fields. We have employed the Drosophila eye model to understand the genetic circuitry responsible for defining the boundary between the eye and the head cuticle on the ventral margin. The default state of the early eye is ventral and depends on the function of Lobe (L) and the Notch ligand Serrate (Ser). We identified homothorax (hth) as a strong enhancer of the L mutant phenotype of loss of ventral eye. Hth is a MEIS class gene with a highly conserved Meis-Hth (MH) domain and a homeodomain (HD). Hth is known to bind Extradenticle (Exd) via its MH domain for its nuclear translocation. Loss-of-function of hth, a negative regulator of eye, results in ectopic ventral eye enlargements. This phenotype is complementary to the L mutant phenotype of loss of ventral eye. However, if L and hth interact during ventral eye development remains unknown. Here we show that (i) L acts antagonistically to hth, (ii) Hth is upregulated in the L mutant background, and (iii) MH domain of Hth is required for its genetic interaction with L, while its homeodomain is not, (iv) in L mutant background ventral eye suppression function of Hth involves novel MH domain-dependent factor(s), and (v) nuclear localization of Exd is not sufficient to mediate the Hth function in the L mutant background. Further, Exd is not a critical rate-limiting factor for the Hth function. Thus, optimum levels of L and Hth are required to define the boundary between the developing eye and head cuticle on the ventral margin.

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Introduction

Axial patterning, which is crucial for the growth of multi-cellular organisms, involves the progressive restriction of cell fate by division of a homogenous group of cells into several subgroups or compartments. The selective spatio-temporal expression pattern of the cell fate selector genes results in the formation of compartments (Curtiss et al., 2002; Dahmann et al., 2011). Complex signaling events between cells of two different compartments promote proliferation and differentiation. Thus, axial patterning, which initially begins with the assignment of compartment specific fates, later contributes towards the transition of a homogeneous group of cells into a three-dimensional organ.

The adult eye of Drosophila develops from an epithelial bi-layer called the eye-antennal imaginal disc (Ready et al., 1976; Wolff and Ready, 1993). The embryonic eye-antennal primordium is a complex disc and is composed of cells derived from several head segments (Younossi-Hartenstein and Hartenstein, 1993). The eye-antennal disc grows and divides into eye and antennal field during larval development (Kenyon et al., 2003; Kumar and Moses, 2001). The developing eye imaginal disc comprises of two different layers viz., the peripodial membrane (PM) and the disc proper (DP). The DP gives rise to the Drosophila retina whereas the PM forms the head cuticle surrounding the eye (Atkins and Mardon, 2009; Cho et al., 2000; Kumar, 2011). Strict genetic regulation decides the size of the eye and its surrounding head cuticle, and this leads to the generation of the eye field boundary.

The Drosophila adult eye is a highly precise hexagonal array of ~800 ommatidial clusters or unit eyes. Each ommatidium has a honeycomb like hexagonal organization and comprises of eight photoreceptor
neurons that are assembled in an asymmetrical trapezoidal pattern (Wolff and Ready, 1993). The ommatidial clusters are arranged in two chiral forms, which are arranged in mirror image symmetry along the Dorso-Ventral (DV) midline called the equator. The eye-antennal imaginal primordium begins from a group of ~20 progenitor cells (Garcia-Bellido and Merriam, 1969; Pousson, 1950; Yamamoto, 1996). The border between the dorsal and the ventral eye compartment, the equator, is the site of activation of Notch (N) signaling, which is responsible for cell proliferation and differentiation in the developing eye disc (Cho and Choi, 1998; Dominguez and de Celis, 1998; Papayannopoulos et al., 1998; Singh et al., 2005b).

The early eye primordium has a default ventral fate, which depends on the function of L and Ser (Oros et al., 2010; Singh et al., 2005b; Singh and Choi, 2003). Later, with the onset of expression of the GATA family zinc finger transcription factor pannier (pnr), the dorsal fate is established over the default ventral eye fate in a subset of eye primordium cells (Dominguez and Casares, 2005; Oros et al., 2010; Singh et al., 2005b; Singh and Choi, 2003). Pnr acts upstream of Wingless (Wg), which in turn induces the expression of members of Iroquois Complex (Iro-C) genes viz., araucan (ara), caupolican (caup) and mirror (mirr). Iro-C genes act downstream of pnr and wg, and are expressed in the dorsal half of the developing eye imaginal disc. Iro-C genes are required for assigning dorsal eye fate and triggering Notch pathway in the DV boundary of the eye (Cavodeassi et al., 1999; Cho and Choi, 1998; Dominguez and de Celis, 1998; Papayannopoulos et al., 1998; Singh et al., 2005b). pnr is expressed in the peripodial membrane on the dorsal margin of the eye disc (Oros et al., 2010; Pichaud and Casares, 2000). Recent studies have demonstrated that Pnr suppresses the eye fate and thereby defines the boundary between the head cuticle and the dorsal margin of the developing eye field (Oros et al., 2010). Since Pnr is expressed only in the dorsal eye margin therefore, pnr is not involved in genetic mechanism regulating the developing eye field boundary on the ventral margin. Thus, the genetic mechanism regulating the boundary of eye field on the ventral margin remains unclear.

In the ventral eye, the loss-of-function of homothorax (hth), results in eye enlargements or ectopic eyes (Pai et al., 1998; Pichaud and Casares, 2000). hth encodes a homeodomain transcription factor of the three-amino-acid extension loop (TALE) subfamily with extensive amino acid identity to the murine proto-oncogene Meis1 (Moskow et al., 1995; Rieckhof et al., 1997). Even though hth is expressed uniformly anterior to the furrow both in the dorsal and the ventral half of the eye, loss-of-function clones exhibit enlargements only in the ventral half of the eye whereas the clones in the dorsal half of the eye do not exhibit any eye phenotypes (Pai et al., 1998; Pichaud and Casares, 2000). However, misexpression of hth suppresses the eye irrespective of the dorsal or the ventral fate. Thus, hth is known to act as the negative regulator of eye development (Pai et al., 1998). Hth has a nuclear localization signal (NLS) and two conserved domains: the N terminal evolutionarily conserved Meis1 (Chern and Choi, 2002), and exons. The MH and HD domains are encoded by exons 2 and 3 respectively. L and Hth interaction is mediated by a novel mechanism that requires the MH domain of Hth but does not require Exd.

In Drosophila, the sub-cellular localization of another homeoprotein extradenticle (Exd) is tightly regulated by Hth. In the absence of Hth, Exd is localized in the cytoplasm, while in the presence of Hth, Exd forms a heterodimer with Hth through its MH domain and translocates into the nucleus to regulate transcription (Abu-Shaar et al., 1999; Aspland and White, 1997; Jaw et al., 2000; Stevens and Mann, 2007). Hth and Exd are also involved in forming a heterodimer with other HOX proteins that alter their DNA binding specificity in the nucleus (Mann, 1995; McGinnis and Krumlauf, 1992). Hth and Exd are involved in a direct protein–protein interaction that is mediated through the N-terminal MH domain. In the eye, Exd is uniformly expressed. However, Exd is nuclear only in the domains where Hth is expressed (Mann and Abu-Shaar, 1996; Rieckhof et al., 1997; Stevens and Mann, 2007), which is the region of the eye disc that develops into the head cuticle surrounding the compound eye (Pai et al., 1998). Thus, Hth and Exd promote head specific fate.

Here we address how L, a gene required for ventral eye development and survival, interacts with hth to control ventral eye growth. We found that antagonistic interaction between L and hth is responsible for defining the size and boundary of the eye field on the ventral margin. Further, L and hth interaction is mediated by a novel mechanism that requires the MH domain of Hth but does not require Exd.

Materials and methods

Fly stocks used are described in Flybase (http://flybase.bio.indiana.edu). We used the following L mutants in this study: L−/−; FRT42D/Cyo, L+/Cyo, L−/Cyo (Chern and Choi, 2002; Singh and Choi, 2003), and UAS-L RNAi (available at VDRC. http://stockcenter.vdrc.at/control/main). L−/− is a null allele of L. (Chern and Choi, 2002), L2 is a dominant negative allele (Singh et al., 2005a), and L+ is a hypomorph (Chern and Choi, 2002). The L alleles used in this study are: hthΔ−/−, hthΔ−/− and hthΔ−/− (Kurant et al., 2001; Noro et al., 2006; Pai et al., 1998). hthΔ−/− is a strong hypomorph generated by P-element insertion in hth promoter (Pai et al., 1998). hth consists of 16 annotated exons. The MH and HD domains are encoded by exons 2–6 and 11–13, respectively. Hth100−/− is predicted to encode only HD-less isoforms due to an Arg321 to opal mutation in exon 9 (Kurant et al., 1998; Noro et al., 2007). hthΔ−/− is a P-element insertion line that serves as an excellent reporter for hth expression in the eye imaginal disc (Pai et al., 1998; Salzberg et al., 1997).

We used the Gal4/UAS system for the targeted misexpression studies (Brand and Perrimon, 1993). We used ey-Gal4 (Hazelett et al., 1998) to drive expression of the transgene in the developing eye field for the gain-of-function studies (Singh et al., 2005a). Various UAS-transgenes used in this study are: UAS-EN-HTH−/− or UAS-EN-HTHΔ−/− a dominant negative allele of hth, generated by fusing the Drosophila EN repression domain (Han and Manley, 1993) to a truncated form of Hth (amino acids 1–430) (Inbal et al., 2001). UAS transgenes harboring the full length hth (hth-FL), and transgenes lacking either Homeodomain (ΔHD) or the Meis Homothorax domain MH (ΔMH) were used for targeted misexpression studies (Jaw et al., 2000; Ryoo et al., 1999). All Gal4/UAS crosses were done at 18 °C 25 °C and 29 °C, unless specified, to sample different induction levels.

Genetic mosaic analysis

We employed genetic mosaic approach to generate loss-of-function clones in the eye (Xu and Rubin, 1993). For the generation of clones in the eye, we have used eyFLP (Newsome et al., 2000) as source of flippase. To generate mosaic clones of (i) L in the eye, eyFLP, FRT42D ubi-GFP virgins were crossed to males of L−/−FRT42D/Cyo, (ii) hth in the eye, eyFLP; FRT82B ubi-GFP virgins were crossed to y, w; FRT 82B hth−/− or FRT 82B hth−/−/TM6B males. Mutant tissue was marked by the absence of GFP reporter.

Immunohistochemistry

Eye-antennal imaginal discs were dissected from wandering third instar larvae and stained following the standard protocol (Singh et al., 2005b).
Antibodies used were rat anti-Elav (1:100), mouse anti-Wg (1:50) (Developmental Studies Hybridoma Bank), rabbit anti-Dlg, anti-Hth (H. Sun and R. Mann), rabbit anti-Exd (Aspland and White, 1997; Mann and Abu-Shaar, 1996), and rabbit anti-Mirr (1:200). Secondary antibodies (Jackson Laboratories) used in this study were goat anti-rat IgG conjugated to Cy5 (1:200), donkey anti-rabbit IgG conjugated to Cy3 (1:250), donkey anti-rat IgG conjugated to FITC, and donkey anti-mouse IgG conjugated to Cy5 (1:200). Immunofluorescent images were analyzed using the Olympus Fluoview 1000 Laser Scanning Confocal Microscope.

Results

hth is a modifier of L in the ventral eye

The L gene function is required for ventral eye development and growth (Chern and Choi, 2002; Singh and Choi, 2003). Loss-of-function of L results in the selective loss of ventral eye in the larval eye imaginal disc (Fig. 1D) and the adult eye (Fig. 1C) as compared to the wild-type eye (Figs. 1A, B). We have identified hth as a modifier of this L mutant eye phenotype of selective loss-of-ventral-eye. Increasing levels of hth gene function in the L mutant eye imaginal disc using gain-of-function approach (L2/+; ey-hth), results in the enhancement of ventral eye loss to a "no-eye" phenotype as seen in the third instar larval eye imaginal disc (Fig. 1H) and the adult eye (Fig. 1G). Loss of eye fate as a result of induction of Hth (L2/CyO; ey-hth) is due to eye to cuticle fate change. Thus, increasing levels of hth gene function enhances the L mutant phenotype in the eye suggesting that hth acts as a genetic modifier of L mutant.

Therefore, we explored the mechanism by which Hth modified the L mutant phenotype of loss-of-ventral-eye. First, we tested if loss of L affects hth expression in the ventral eye. In the developing third instar eye imaginal disc, Hth is strongly expressed anterior to the furrow, which corresponds to the region that forms the pitilium, ocellus, head capsule, and also in the posterior and lateral margins of the eye disc (Fig. 1B). Hth is expressed in the cells of the peripodial membrane of the eye disc and weakly in the posterior region that is composed of mature photoreceptors (Bessa et al., 2002; Pai et al., 1998, 1999; Pichaud and Casares, 2000; Singh et al., 2002). Even though Hth is a transcription factor that needs to be localized in the nucleus, it is present both in the cytoplasm as well as the nucleus whereas L is located in the cytoplasm (data not shown). We found that in L mutant background Hth expression was upregulated (Fig. 1D; arrow). Since the majority of cells in the ventral half of the eye are lost in the L2/+ mutant eye imaginal disc (Singh et al., 2006), Hth upregulation was seen only on the ventral margin (Fig. 1D, arrow). However, there is a need to verify if it is an additive effect or a real interaction since increasing levels of hth alone in the eye (ey-hth) results in the suppression of eye (Pai et al., 1998; Singh et al., 2002). Hth is known to be a negative regulator of the eye (Pai et al., 1998). In order to test the genetic interaction between L and hth, we decided to analyze their loss-of-function phenotypes in the eye.

L and hth exhibit complementary loss-of-function phenotype in eye disc

Loss-of-function clones of L in the eye exhibit domain specific phenotype. Loss-of-function clones of L in the ventral eye result in the selective loss of eye fate (Figs. 2A, A′) as evident from suppression of neural marker ELAV (Fig. 2A′). However, in the dorsal eye these clones have no effect on the eye fate. Interestingly, loss-of-function clones of hth in the ventral eye result in eye enlargement or induction of ectopic eye (Pai et al., 1998) whereas in the dorsal eye these clones do not affect the eye fate (Figs. 2B, B′, arrow). Thus, hth loss-of-function clones also exhibit a dorsal–ventral constraint in their phenotypes. Given the opposing outcomes of hth and L loss-of-function on the ventral eye fate, we further explored the interaction of L and hth by testing the expression of Hth in the L mutant cells in the eye imaginal disc. Interestingly, both L and hth are not expressed in a domain specific manner during eye development (Bessa et al., 2002; Singh and Choi, 2003).

**Fig. 1.** hth acts as a modifier of the L mutant phenotype of preferential loss-of-ventral-eye. (A, B) Wild-type adult eye and eye imaginal disc are shown. The border between the dorsal (D) and the ventral (V) compartment of the eye marks the equator. (B) Hth (green) is expressed only anterior to the morphogenetic furrow (MF) in the eye disc, which corresponds to the adult head cuticle. Elav (red), a pan neural marker, marks the photoreceptors in the eye. (C, D) L2/+ mutant exhibits preferential loss-of-ventral-eye phenotype in the eye disc (marked by the dotted line) and adult eye. (D) L2/+ mutant eye disc exhibits strong induction of Hth (green) on the ventral eye margin. Since the majority of ventral eye is lost in the third instar eye disc, we can see ectopic Hth expression only on the ventral margin (arrow). (E, F) Misexpression of hth in the entire eye using the ey-GAL4 driver (ey-hth) suppresses the eye fate in the eye imaginal disc and the adult eye. (G, H) Misexpression of hth in the L2/+ mutant eye background (L2/+; ey-hth) results in strong enhancement of loss-of-ventral-eye phenotype to a "no-eye" phenotype as evident from absence of any Elav positive cells in the eye disc. Note that there is a change in eye to head cuticle fate. All images are oriented as dorsal (up), ventral (down), anterior (right), and posterior (left).
Hth is ectopically induced in the L mutant clones

The loss-of-function clones of L in the ventral eye exhibit a loss of eye fate based on the absence of the pan-neural marker Elav, which marks the photoreceptor specific fate (Figs. 3B, B′–B″). In comparison to wild-type Hth expression in the eye disc (Fig. 3A), these loss-of-function clones of L in the ventral eye showed robust induction of Hth expression (Figs. 3B, B′ clone boundary marked by white dotted line, inset shows Hth upregulation in ventral eye clone) whereas the dorsal clones do not effect the eye fate or the Hth expression (Figs. 3C, C′, C″ clone boundary marked by white dotted line). We have counted 51 L loss-of-function clones. The distribution of these clones is 42 in the dorsal eye and 9 in the ventral eye. The dorsal clones did not show any effect on eye fate as well as Hth expression. The 9 ventral clones showed ectopic Hth induction and concomitant loss of eye fate. The discrepancy in the number of dorsal versus ventral clones is because of the fact that L mutant clones in the ventral eye do not survive (Singh et al., 2006). We further tested this interaction using an enhancer trap line where the lacZ reporter gene is expressed under the hth promoter. Because the mutant L′′ eye discs show a complete loss of the ventral eye, we tested the expression of hth-reporter in a hypomorphic L mutant LΔ4, where the heterozygous eye has an anterior nick in the eye or wild-type eye (Chern and Choi, 2002). Interestingly, this hth reporter showed ectopic expression in the ventral margin of the eye imaginal disc in the heterozygous L (LΔ4/+) mutant background (Fig. 3D; arrow). Next, we tested the L and hth interaction using L RNAi. Misexpression of UAS-L RNAi in the eye using ey-Gal4 (ey-L RNAi) resulted in a highly reduced eye field where ventral half of the eye is lost along with upregulation of Hth on the ventral eye margin (Fig. 3E). Thus, any loss of eye fate in the L loss-of-function clones is associated with the induction of Hth. Interestingly, L and hth interaction seems to exhibit a domain constraint based on the restriction of their loss-of-function phenotypes only to the ventral eye even though they are expressed both in the ventral and the dorsal eye (Bessa et al., 2002; Pai et al., 1998; Singh et al., 2002).

L acts antagonistically to hth

We analyzed genetic interactions between these two genes. We found that reducing the levels of hth to half in the L mutant background (LΔ2/+; hthΔ422–4/+ ) exhibits a partial rescue of the L mutant phenotype of loss-of-ventral-eye (Fig. 1C) in the eye imaginal disc (Figs. 3F, G) as well as the adult eye (Fig. 3H). We employed a dorsal fate marker, Mirr expression to show the rescue of the ventral eye (Fig. 3G). We also tested this interaction by misexpressing UAS-hthΔNR, the dominant negative allele of hth in the L mutant eye disc (L′, ey-hthΔNR). The repressor form of Hth was generated by fusing the Drosophila EN repression domain (Han and Manley, 1993) upstream to a truncated form of Hth (amino acids 1–430; EN-HthΔENR) (Inbal et al., 2001). We found that the misexpression of UAS-hthΔNR in L mutants (LΔ2; ey-hthΔNR) caused a significant rescue of the loss of ventral eye phenotype in the eye imaginal disc (Figs. 3I, J) as well as the adult eye (Fig. 3K). We also tested whether the rescue was due to growth of the ventral eye by using Mirr expression as a marker for the dorsal fate. We found that dorsal specific expression of Mirr was restricted only to the dorsal half and there was a significant rescue of the ventral eye fate (Fig. 3J). Thus, reducing Hth levels can rescue the L mutant phenotype in the ventral eye. On the contrary, increasing the levels of hth in the L mutant eye imaginal disc (LΔ2/+; ey-hth) enhances the loss of ventral eye phenotype to a “no-eye” phenotype (Figs. 1G, H). There was no effect on the antennal field. Thus, a reduction or increase in the levels of hth in the L mutant eye disc has converse effects on the loss-of-ventral-eye phenotype. Our results clearly suggest that L genetically interacts with hth in the ventral eye and this interaction is antagonistic in nature (Fig. 3L).

L requires MH domain of Hth for its interaction in the eye

Since we found that L acts antagonistically to hth (Fig. 3), we next focused on identifying the domain of Hth that interacts with L. Hth encodes a protein with an evolutionarily conserved MH domain and a DNA binding homeodomain (Fig. 4A) (Inbal et al., 2001; Jaw et al., 2000; Ryoo et al., 1999). To test the domain specific requirement of Hth for its interaction with L, we used transgenic constructs that misexpress truncated forms of Hth to study their effect on the L mutant phenotype (Fig. 4A; Jaw et al., 2000; Ryoo et al., 1999). We tested individually the MH domain and the homeodomain of Hth for their requirement in interaction with L in the eye using the gain-of-function approach. Misexpression of ΔMH domain of hth in the eye (ey-hthΔMH) does not affect the eye size (Fig. 4B) whereas misexpression of ΔHD (ey-hthΔHD) results in suppression of the eye (Fig. 4C). In L mutant eye imaginal disc, overexpression of the hth transgene lacking only the MH domain (LΔ2; ey-hthΔMH) did not affect the loss-of-ventral-eye phenotype of the L mutant as seen in the eye imaginal disc (Fig. 4F) as well as the adult eye (Fig. 4D). However, when we misexpressed the hth construct lacking the homeodomain (HD) in the L mutant eye background (LΔ2; ey-hthΔHD), it resulted in a “no-eye” phenotype in the eye imaginal disc as well as the adult eye (Figs. 4E, G). These phenotypes are comparable to the ones seen with misexpression of the full length hth transgene in eye imaginal disc and the adult eye (Figs. 1G, H). These results suggest that the MH domain of Hth is crucial for its antagonistic interaction with L.

L interacts with the alternative splice variant of hth with only the MH domain

In this study we used the two different alternative spliced variants of hth, one with the HD domain and the other without HD (Noro et al., 2006). To address the function of MH domain in vivo, we utilized the hth100-1 mutant that results in a HD-less form of Hth (Fig. 5A; Noro et al., 2006). We found that the loss of function of hth using the null allele results in ventral eye enlargement as
seen in the adult and the eye imaginal disc (Figs. 5B, C; Pai et al., 1998; Pichaud and Casares, 2000). Interestingly, when we generated loss-of-function clones of \( L \) in the heterozygous background of the \( hth \) null allele (\( L^{-/-}; hth^{-/-} \)), they did not show any suppression of the eye fate in the adult (Fig. 5F). This phenotype is different from \( L \) loss-of-function clone phenotypes (\( L^{-/-} \)) of loss of ventral eye (Figs. 2A; 3A). Loss-of-function clones of \( hth^{100-1} \) did not show any significant ventral eye enlargement or ectopic ventral eye in the adult (Fig. 5D) or the eye imaginal disc (Fig. 5E). However, when we generated \( L \) loss-of-function clones in the heterozygous background of \( hth^{100-1} \) (\( L^{-/-}; hth^{100-1/-} \)), these clones resulted in complete loss-of-ventral-eye (Fig. 5G) as seen in the \( L \) loss-of-function clones (Fig. 2A). These results further validated that the highly conserved MH domain of \( hth \) is crucial for its antagonistic interaction with \( L \). However, the HD is dispensable for \( L \) and \( Hth \) interaction. Interestingly, the same MH domain of \( Hth \) is required for its interaction with Exd in the eye. Therefore, we tested if \( L \) interacts with \( Hth \) through Exd in the ventral eye.

**Fig. 3.** \( L \) interacts antagonistically with \( hth \). (A) Wild-type expression of \( Hth \) (red) in the eye imaginal disc. Dlg (green) marks the membrane and Elav (blue) marks the photoreceptor neuron fate. (B–B′) Loss-of-function clone of \( L \), which shows selective loss-of-ventral-eye fate as evident from the loss of Elav (blue) positive cells, is also accompanied with the ectopic induction of \( Hth \) (red). The insets in B–B′ show a magnified view of the ventral clone. (C–C′) Loss-of-function \( L^{-/-} \) clones in the dorsal eye (clone boundary marked by the dotted line) had no effect on the eye fate and lacked any ectopic induction of \( Hth \) in the eye. (D) Misexpression of UAS-\( L \) RNAi (\( ey\)-LRNAi) results in suppression of the eye fate with ectopic induction of \( Hth \) (green) on the ventral eye margin. (E) A lacZ reporter under the \( hth \) promoter which is expressed in a specific domain anterior to the MF in the developing eye imaginal disc shows ectopic induction in the ventral eye in the \( L^{-/-} \) heterozygous background marked by an arrow. (F–H) Reducing the levels of \( hth \) function to 50% using a null allele \( hth^{1422-4} \) in the \( L^{2/-} \) heterozygous background (\( L^{2/-}; hth^{1422-4/-} \)), results in the partial rescue of the loss-of-ventral-eye phenotype. (F, G) Reducing the \( hth \) function by dominant-negative \( hth^{ENR} \) in the \( L^{-/-} \) mutant background (\( L^{-/-}; ey\)-\( hth^{ENR} \)) results in the significant rescue of the loss-of-ventral-eye phenotype in the (F) eye imaginal disc and the (G) adult eye. (H) \( L \) antagonizes \( Hth \) in the ventral eye. Interestingly, this interaction does not hold true in the dorsal eye even though both \( L \) and \( Hth \) are expressed in the dorsal eye.

\( L \) does not interact with Exd to define the ventral eye margin

\( Hth \) is known to form a heterodimer with Exd and the resultant complex moves to the nucleus to regulate transcription of the target genes (Abu-Shaar et al., 1999; Aspland and White, 1997; Jaw et al., 2000; Pichaud and Casares, 2000). Interestingly, when we generated loss-of-function clones of \( L \) in the heterozygous background of the \( hth \) null allele (\( L^{-/-}; hth^{-/-} \)), they did not show any suppression of the eye fate in the ventral eye (Fig. 3A). Loss-of-function clones of \( hth^{100-1} \) did not show any significant ventral eye enlargement or ectopic ventral eye in the adult (Fig. 5D) or the eye imaginal disc (Fig. 5E). However, when we generated \( L \) loss-of-function clones in the heterozygous background of \( hth^{100-1} \) (\( L^{-/-}; hth^{100-1/-} \)), these clones resulted in complete loss-of-ventral-eye (Fig. 5G) as seen in the \( L \) loss-of-function clones (Fig. 2A). These results further validated that the highly conserved MH domain of \( hth \) is crucial for its antagonistic interaction with \( L \). However, the HD is dispensable for \( L \) and \( Hth \) interaction. Interestingly, the same MH domain of \( Hth \) is required for its interaction with Exd in the eye. Therefore, we tested if \( L \) interacts with \( Hth \) through Exd in the ventral eye.

\( L \) does not interact with Exd to define the ventral eye margin

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It is possible that L might prevent Hth–Exd binding in the cytoplasm. Therefore, we tested whether L–Hth interaction also requires Exd or is independent of Exd function. Exd is present in the cytoplasm in the eye imaginal disc, but Exd localization becomes nuclear only where Hth protein is present (Figs. 6A, A′, A″). It has been shown that Exd is functional only when it is localized in the nucleus (Mann and Abu-Shaar, 1996; Rieckhof et al., 1997; Stevens and Mann, 2007). To test whether L interacts with ventral eye, we generated L loss-of-function clones in the eye and tested the expression of Exd. The L loss-of-function clones in the ventral eye showed strong ectopic nuclear localization of Exd along with a loss of Elav (Figs. 6B–B″, inset). These results further suggest that either L interacts antagonistically with both exd and hth or with hth alone. Therefore, we tested epistatic interactions between L and exd. The rationale of the experiment was if L and Exd interact antagonistically to each other, then reducing exd function will rescue the L mutant phenotype. In the L mutant heterozygous background that exhibits loss-of-ventral-eye, we further reduced the exd gene function (exd<sup>−/+</sup>; L<sup>−</sup>/+), and found that the L loss-of-ventral-eye phenotype remains unaffected (Fig. 6C). Conversely, we overexpressed exd in the L<sup>−</sup> mutant eye background (L<sup>−</sup>; ey-exd) and found that the L mutant phenotype of loss-of-ventral-eye was not affected (Fig. 6D). We also generated L loss-of-function clones in an exd heterozygous background but found no effect on the L loss-of-function clone phenotype of loss-of-ventral-eye (Fig. 6E). These results suggest that L and exd may not interact with
each other or Exd is not a rate-limiting factor for the Hth function in the L mutant background. Therefore, in order to understand the nuclear localization of Exd in L mutant clones in the ventral eye, we tested the expression of both Hth and Exd. We found that in loss-of-function clones of L in the ventral eye, both Hth and Exd were ectopically localized in the nucleus (Figs. 6G–G‴). Note that G‴′–G‴ are the magnified views of the clone. Thus, Exd nuclear localization in the L loss-of-function clones may be due to ectopic induction of Hth. It is known that Hth can form a complex with Exd and drive the hetero-dimer complex to the nucleus. We tested this hypothesis by making the L loss-of-function clones in a heterozygous background of hth null allele (L′/+; hth−/+; ey>ey) and observed that there was no ventral eye loss and Exd was no longer nuclear in these clones (Fig. 6F). Thus, L interaction with hth may not solely depend on nuclear Exd localization.

Discussion

During organogenesis, axial patterning plays a crucial role in transition of a monolayer of primordium cells into a three-dimensional organ. One of the interesting facets of patterning is constant refinement of a large multipotent developing field into smaller fields by progressive restriction of cell fates. These smaller subfields within a developing field are called compartments (Curtiss et al., 2002;
However, there are some interesting questions pertaining to this complex process of sequential restriction of cell fates. For example (i) how are the new compartment boundaries laid within a developing field comprising of a homogenous cell population? (ii) What decides where the boundary will be established within a single or two adjoining developing fields? *Drosophila* eye serves as an excellent model to address these questions of positional fate restrictions as the genetic circuitry involved in retinal determination, axis determination and genes involved in negative regulation of eye fate are known. In this study, we investigated the mechanism responsible for generating the boundary between the developing eyes versus the head field on the ventral eye margin. Interestingly, both head cuticle and eye field are generated from the same eye-antennal imaginal disc, which begins as a homogenous group of cells in the eye primordium. Thus, further assignment of developmental fates within the eye field by differential regulation of gene expression, will result in delineation of eye versus head fate (Kenyon et al., 2003; Kumar and Moses, 2001). Although the genes involved in eye versus head fate are known but how does their interaction fine tune the boundary between the head versus eye fields is not clear.

In *Drosophila* eye, DV patterning, an essential component of axial growth, is the first lineage restriction event (Singh et al., 2005b; Singh and Choi, 2003). DV patterning results in the generation of dorsal and ventral compartments in the eye (Dominguez and Casares, 2005; Singh et al., 2005b). In *Drosophila*, ventral is the default state of early eye primordium. The default ventral eye fate depends on the function of the *L* gene (Singh et al., 2005b; Singh and Choi, 2003). The homogenous group of cells of early eye primordium with ventral fate gets divided into two different dorsal and ventral fates after the onset of expression of dorsal selector genes. The boundary between the dorsal and ventral compartments is crucial for the growth of eye as an organ.

There is also a boundary between the eye field and the prospective head cuticle. Previously, we have shown that the boundary between developing eye field and the head cuticle on the dorsal margin is regulated by *pnr* gene function (Oros et al., 2010). However, *pnr* is not expressed in the ventral eye. Therefore, a different genetic mechanism might be in place to generate the boundary between the head field and the head cuticle on the ventral margin. Here, we have focused on the question pertaining to the delineation of the boundary between the head cuticle and the developing eye field on the ventral margin (Fig. 7).

The *Drosophila* eye primordium begins from the ventral eye on which the dorsal eye fate is established. *L* plays a role in ventral eye development, growth and survival. Loss-of-function of *L* results in preferential loss of ventral eye (Figs. 1, 2). We found that *hth*, a modifier of *L* mutant phenotype in the ventral eye (Fig. 1), exhibits ventral specific function. Loss-of-function of *hth* results in enlargement of the eye on the ventral margin of the developing eye field (Fig. 2). Thus, *L* and *hth* exhibit complementary loss-of-function phenotype, and may act antagonistically to each other (Fig. 3). This conclusion is based on (i) ectopic induction of *Hth* in the loss-of-function clones of *L*, (ii) reducing *hth* gene function, either by a classical mutant approach or by using dominant negative strategy, rescues the *L* mutant phenotype of loss-of ventral eye (Fig. 3), and (iii) enhancing *hth* gene function enhances the *L* mutant phenotype of loss-of ventral eye to a “No-eye” (Fig. 1).

**Fig. 7.** Antagonistic interactions of *L* with *hth* define the boundary between the head cuticle and the developing eye field on the ventral margin. *L*, a gene required for ventral eye development, interacts antagonistically with the dorsal eye selector *pnr* to define the equator (Singh et al., 2005a). Equator is the boundary between the dorsal and ventral compartments in the eye. This study shows that the boundary between the eye field on the ventral margin and the head cuticle depends on the antagonistic interaction between *L* and *hth*. The fine tuning of the levels of *L* and *hth* is crucial to define the boundary of eye field on ventral margin. Interestingly, *exd* may not be critical for the antagonistic interactions between *L* and *hth* in the ventral eye. *Exd* forms a heterodimer with *Hth* and resultant *Hth–Exd* dimer is transported to nucleus. It is known that *Hth–Exd* dimer present in nucleus suppresses the eye fate.

**Optimum levels of *L* and *hth* define the boundary of eye and head on ventral margin**

Our studies show that the fine tuning of optimal levels of *L* and *Hth* defines the boundary of the eye on the ventral margin. Under wild-type conditions, *L* promotes ventral eye development (Chern and Choi, 2002; Singh et al., 2005b; Singh and Choi, 2003) whereas *hth* promotes the head cuticle fate on the ventral eye margin (Pai et al., 1998; Pichaud and Casares, 2000). However, there is no information available about their mutual interaction. Our study demonstrates that *L* acts antagonistically to *hth* (Figs. 3, 7). Therefore, the size of the eye field in the ventral domain is an outcome of fine tuning of balance in *L* and *hth* levels. If the balance shifts in favor of *hth* (*L* mutant background), it results in the loss-of-ventral-eye whereas in converse situation where balance shifts away from *hth* (*hth* mutant background), it results in the enlargement of the ventral eye domain (Fig. 7).

$L$ promotes ventral eye development by suppressing Wg signaling (Singh et al., 2006). Wg is known to act as a negative regulator of eye (Pichaud and Casares, 2000; Treisman and Rubin, 1995). Ectopic upregulation of Wg signaling in the *L* mutant background results in the loss of ventral eye (Singh et al., 2006). However, it is not clear how *L* regulates Wg signaling to regulate ventral eye development. Wg is expressed strongly in the dorsal eye margin as compared to the ventral eye margin. Removal of Wg in the dorsal eye results in ectopic furrow with similar results in ventral, however with less penetrance (Pichaud and Casares, 2000; Treisman and Rubin, 1995). Wg regulation in the dorsal and the ventral eye is different. In the dorsal eye, Wg acts downstream of Pnr (Maurel-Zaffran and Treisman, 2000). In the ventral eye, hth maintains Wg, and they act in a positive feedback loop to suppress the eye fate (Pichaud and Casares, 2000; Singh et al., 2005b). We have found that *L* and *hth* interact antagonistically to each other. Therefore, the genetic interaction of *L* and Wg in the ventral eye (Singh et al., 2006) may be mediated through Hth. Hth, Teashirt (Tsh) and PAX-6 homolog Eyesless (Ey) are coexpressed in a region anterior to the morphogenetic furrow and their complex is responsible for cell proliferation (Bessa et al., 2002; Lopes and Casares, 2010). We have earlier shown that Tsh and *L* do not interact (Singh et al., 2004). Furthermore, *L* may act downstream of ey (Singh unpublished data). Therefore, in light of these evidences *L* and *Hth* interaction may be exclusive.

$L$ interacts with MH domain containing alternative spliced variant of *Hth*

Hth is known to form two different alternative spliced variants (Glazov et al., 2005; Noro et al., 2006). Our studies on domain
requirement suggested that evolutionarily conserved MH domain of Hth is crucial for its interaction with L mutant phenotype (Fig. 4). We found that misexpression of transgene encoding truncated Hth protein lacking MH (Hth ΔMH) domain does not affect the L mutant phenotype of ventral eye loss whereas the misexpression of transgene encoding Hth protein lacking HD (Hth ΔHD) enhances the L mutant phenotype of loss of ventral eye to “no-eye”. In fact, the effect of misexpression of Hth ΔHD was similar to Hth100-1 (HD-less), an alternative spliced variant of Hth, which does not have a homeodomain. Since MH domain of Hth is required for its interaction with Exd, we tested interaction of L with Exd.

**Exd may not be a critical factor for L and Hth interaction in the ventral eye**

Hth is required for nuclear localization of Exd. Exd forms a heterodimer with Hth, and Hth–Exd heterodimer is then shuttled to the nucleus to carry out its function. Exd is functional only when it is present in the nucleus (Aspland and White, 1997; Mann and Abu-Shaar, 1996). Hth is required for Exd nuclear localization and function whereas Hth requires Exd for its stability. It has been shown that some of the functions require both Hth–Exd whereas some only require nuclear Exd. Both Hth and Exd loss-of-function show similar phenotype in the eye thereby suggesting both are required for eye development. Therefore, we tested whether L interacts with hth or with Hth–Exd complex to define the ventral eye margin. Interestingly, we found that L–Hth interaction to define the margin of the ventral eye may work by a novel mechanism which is not critically dependent of Exd (Fig. 6). Our conclusions were supported by the results from our experiment where L mutant phenotype in the ventral eye was rescued by misexpression of dominant negative Hth (hthΔN). It has been shown that dominant negative Hth (hthΔN) does not interfere with the nuclear localization of Exd and that it is capable of driving Exd into the nucleus (Inbal et al., 2001). Thus, nuclear localization of Exd is not sufficient to mediate the Hth function in the L mutant background. Furthermore, genetic epistatic analysis of L and exd showed that they do not interact (Fig. 6). These findings suggest that genetic interaction between L and Hth in the ventral eye is independent of Exd or that Exd is not a rate-limiting factor.

Therefore, our results suggest that ventral eye development gene L antagonistically interacts with hth, a negative regulator of eye to define the ventral eye margin (Fig. 7). Surprisingly, L and hth are expressed in both the dorsal and the ventral half of the eye. However, their functional domain (Fig. 2) as well as their antagonistic interaction is restricted only to the ventral half of the eye. It is possible that either the interaction between L and Hth is not direct or there is a factor in the dorsal domain that prevents the interaction of L and Hth in the dorsal half of the eye. It is possible that dorsal selector ptr, which establishes the dorsal fate over the default ventral eye fate, might be that factor. It is reported that loss-of-function of ptr results in enlargement of the dorsal eye (Maurel-Zaffran and Treisman, 2000; Oros et al., 2010).

**L is an ortholog of PRAS40 (Oshiro et al., 2007; Vander Haar et al., 2007; Wang and Huang, 2009) and hth is a Drosophila homolog of MEIS1 that plays an important role in vertebrate eye development (Bessa et al., 2008; Mann and Abu-Shaar, 1996; Moskow et al., 1995; Pai et al., 1998; Rieckhoff et al., 1997). Thus, there is a strong possibility that similar regulatory interactions between L and Hth may occur in the higher organisms that may have implications on the development of field boundaries.**

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Activation of JNK Signaling Mediates Amyloid-β-Dependent Cell Death

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Abstract

Background: Alzheimer’s disease (AD) is an age-related progressive neurodegenerative disorder. One of the reasons for Alzheimer’s neuropathology is the generation of large aggregates of Aβ42 that are toxic in nature and induce oxidative stress, aberrant signaling, and many other cellular alterations that trigger neuronal cell death. However, the exact mechanisms leading to cell death are not clearly understood.

Methodology/Principal Findings: We employed a Drosophila eye model of AD to study how Aβ42 causes cell death. Misexpression of higher levels of Aβ42 in the differentiating photoreceptors of fly retina rapidly induced aberrant cellular phenotypes and cell death. We found that blocking caspase-dependent cell death initially blocked cell death but did not lead to a significant rescue in the adult eye. However, blocking the levels of c-Jun NH(2)-terminal kinase (JNK) signaling pathway significantly rescued the neurodegenerative phenotype of Aβ42 misexpression both in eye imaginal disc as well as the adult eye. Misexpression of Aβ42 induced transcriptional upregulation of puckered (puc), a downstream target and functional read out of JNK signaling. Moreover, a three-fold increase in phospho-Jun (activated Jun) protein levels was seen in Aβ42 retina as compared to the wild-type retina. When we blocked both caspases and JNK signaling simultaneously in the fly retina, the rescue of the neurodegenerative phenotype is comparable to that caused by blocking JNK signaling pathway alone.

Conclusions/Significance: Our data suggests that (i) accumulation of Aβ42 plaques induces JNK signaling in neurons and (ii) induction of JNK contributes to Aβ42 mediated cell death. Therefore, inappropriate JNK activation may indeed be relevant to the AD neuropathology, thus making JNK a key target for AD therapies.

Introduction

Alzheimer’s disease (AD) is the most prevalent neurodegenerative disease, and is characterized by a gradual loss of synapses and neurons in the hippocampus and cortex, leading to a decline in cognitive function and memory [1,2,3,4,5]. The AD brain is typically associated with two types of protein deposits, amyloid plaques and neurofibrillary tangles. The generation of large aggregates of Aβ42 is toxic in nature and induces oxidative stress, aberrant signaling, and many other cellular alterations that trigger neuronal cell death. However, the exact mechanisms leading to cell death are not clearly understood.

The amyloid-β hypothesis proposes that Aβ42 initiates the pathogenic cascade in AD, including aberrant cell signaling and Tau hyperphosphorylation [6]. Thus, understanding how Aβ42 induces neurotoxicity [2,7] and cell death are key questions in AD. Soluble and insoluble Aβ42 assemblies cause multiple alterations to cellular homeostasis, including mitochondrial dysfunction and oxidative stress, misregulation of intracellular calcium, ER stress, and aberrant signaling through interaction with several receptors. Even though a lot has been learned by modeling AD in animal model systems like mouse [2,3] and fruit fly [3,8,9,10,11,12,13,14], the exact mechanisms mediating Aβ42-dependent cell death remain elusive. A hallmark of AD neuropathology as a result of Aβ42 plaques is the death of neurons [3]. Since, several genetic pathways like caspase dependent cell death pathway, P53-dependent cell death [15,16], and c-Jun amino-terminal (NH2)-kinase (JNK) signaling [17,18,19,20] pathways are involved in cell death mechanism, it is possible that some of these pathways may be involved in neurodegeneration observed in Aβ42 plaques.
The *Drosophila* eye serves as an excellent model to study patterning, growth, and is well-suited to study cell death [3,13,14]. The compound eye of *Drosophila* develops from an epithelial bilayer structure present inside the larva and referred to as the eye-antennal imaginal disc. The eye-antennal imaginal disc is a complex disc, which gives rise to an eye, antenna and head cuticle of the adult fly [21,22]. The retinal precursor cells of the eye imaginal disc undergo differentiation to form photoreceptor neurons during the third larval instar [23,24]. Once eye differentiation is complete, the compound eye of the adult fly is comprised of about 800 units called ommatidia, each containing eight photoreceptors and several support cells. In the pupal retina, the excessive cells other than the differentiated cells are eliminated by programmed cell death (PCD) [25]. There is no PCD during earlier stages of larval eye development. However, abnormal extracellular signaling due to inappropriate levels of morphogens may trigger cell death in the developing larval eye imaginal disc [26]. Wingless (Wg), a morphogen, is known to trigger PCD in ommatidia present at the periphery of the pupal retina [27,28] whereas ectopic Wg expression can also induce developmental cell death earlier in the developing larval eye imaginal disc [29]. In *Drosophila*, three pro-apoptotic genes: *head involution defective* (hid), *veaper* (vpe), and *grim* can trigger cell death by negatively regulating *Drosophila* inhibitor of apoptosis (DIAP1) [30,31,32]. DIAPs are the members of a highly conserved class of proteins, which negatively regulate caspase activity [33,34,35]. In response to pro-apoptotic signals, Hid, Rpr and Grim contribute to DIAP1 degradation, leading to the activation of initiator- (Drone/caspase 9) as well as effector-caspases (Drice/caspase 3). Activation of initiator caspase triggers the caspase-dependent cell death. The caspase dependent cell death can be blocked by higher expression levels of baculovirus protein P35 [36]. However, not all the cell death is caspase-dependent. For instance, extrinsic signals like UV-irradiation that cause DNA damage and consequently trigger P53-activated downstream of the Tumor Necrosis Factor (TNF) homologue Eiger (Egr) and its receptor Wengen (Wgn) by a kinase (JNK) signaling pathway can induce caspase-independent cell death [15,37,38].

Activation of the JNK, or stress activated kinase proteins of the mitogen-activated protein kinase (MAPK) super family [17,20,37] may also trigger cell death due to phosphorylation of transcription factors regulating cell death [39]. It has been proposed that activation of JNK signaling leads to induction of cell death to eliminate developmentally aberrant cells, thus ensuring tissue robustness [17,37,40]. In *Drosophila*, JNK signaling pathway is activated downstream of the Tumor Necrosis Factor (TNF) homologue Eiger (Egr) and its receptor Wengen (Wgn) by a conserved signaling cascade that includes Tak1 (TGF-β-activating kinase 1); a JNK kinase kinase (JNKK), Hemipterous (Hep; a JNK kinase), Basket (Bsk; a Jun kinase), and Jun [20,40,41].

The functional readout for the activation of JNK signaling is the expression levels of *puckered* (puc) gene, which encodes a dual specificity phosphatase, and forms a negative feedback loop by down regulating the activity of JNK [17,37,43]. Ectopic activation of JNK signaling has been shown to trigger apoptosis during early eye imaginal disc development [29,44]. Although JNK signaling mediates cell death through *sps* and *hid*, caspase inhibition does not completely prevent JNK-dependent cell death. Thus, JNK regulates apoptosis through caspase-independent mechanisms [39].

Recent observations have linked the JNK pathway to AD, including the ability of JNK to phosphorylate Tau and APP *in vitro*, promoting the accumulation of two neurotoxic species: hyperphosphorylated Tau and Aß42 [18]. Here, we demonstrate the role of JNK signaling in Aß42 neurotoxicity using a *Drosophila* model of AD. In *Drosophila*, misexpression of Aß42 in neurons of the brain resulted in decline in locomotor function, age dependent learning defects, progressive loss of neurons and reduced lifespan [3,10,14]. Here we demonstrate that Aß42 induces aberrant cellular morphology and increased cell death in the developing retina in late third instar eye imaginal disc. We also found that JNK signaling is activated in neurons where Aß42 is misexpressed, suggesting a role for JNK in Aß42-mediated cell death. In fact, activation of JNK signaling exacerbated Aß42 neurotoxicity, whereas downregulation of the JNK pathway prevented cell death and rescued eye size and organization. Furthermore, suppression of both JNK signaling and caspase-dependent cell death led to a suppression of Aß42 neurotoxicity in the eye, which is relatively comparable to the rescue caused by blocking JNK signaling thereby suggesting that JNK signaling mediated cell death plays an important role in AD neuropathology.

**Results**

**Aß42 induces early cellular phenotypes in the developing eye disc**

Aß42 misexpression in the *Drosophila* eye imaginal disc induces strong phenotypes, including reduced eye size, disorganized and fused ommatidia in the adult eye [14,45]. To understand how Aß42 exerts its neurotoxicity in the eye, we followed the early events in the development of the retina upon misexpression of Aß42. For these studies, we used GMR-Gal4 driver [46]. We employed GFP reporter to study spatio-temporal expression profile of GMR-Gal4 driver (GMR>GFP). GMR>GFP drives GFP reporter expression only in the differentiating photoreceptor neurons of the developing third instar larval eye imaginal disc (Figure 1A, A’). The misexpression of Aß42 in the larval eye imaginal discs, detected by 6E10 antibody [47], corresponds to the domain comprising of the differentiating photoreceptors, as indicated by Elav accumulation (Figure 1B, B’). The photoreceptors differentiation occurs in the third instar larval eye imaginal disc (Figure 1C, C’). The high organization of the developing eye can be appreciated by looking at the cell outlines, as indicated by the basal lamina marker Disc large (Dlg) (Figure 1C, C’) [48]. Only a few hours after Aß42 expression starts, the eye territory of the third instar eye imaginal disc exhibits subtle phenotypes. The distribution of the photoreceptors is not normal (Figure 1D and D’), the arrangement of basal membranes as shown by Dlg expression [48] indicates improper spacing (Figure 1D, D’, arrow) of the differentiating photoreceptors and mild fusion of the ommatidial clusters. Since these phenotypes worsen with aging, these abnormal photoreceptor cells are possibly basally extruded later and lost from the disc lamina.

During metamorphosis of larva to a early white pupa (prepupa), the larval eye imaginal disc with differentiated photoreceptor cells undergoes transition to a prepupal retina. Although other cell types along with the photoreceptor neurons are recruited in the prepupal retina the overall morphology still resembles an eye imaginal disc (Figure 1E, E’). A few hours after Aß42 expression, the neurodegenerative phenotypes of the prepupal retina get stronger (Figure 1F). In comparison to the precise arrangement of photoreceptor neurons in wild-type early pupal retina (Figure 1E, E’), the prepupal retina with Aß42 misexpression exhibits mild to stronger fusion of the ommatidial clusters (Figure 1F, F’, arrow) resulting in clumps of photoreceptor neurons and loss of photoreceptors in the ommatidium (Figure 1F, F’, arrow, dotted outline marks the ommatidial clumps).

**Analysis of Aß42 misexpression phenotypes during development**

We followed our investigation of the neurodegenerative phenotype of Aß42 misexpression in the prepupal retina and the adult eye...
Figure 1. Misexpression of amyloid-beta 42 (Aß42) induces cellular phenotypes in the differentiating retinal neurons. (A, A') GMR-Ga4 drives expression of GFP reporter (GMR>GFP) in the differentiating photoreceptor neurons in the third instar eye imaginal disc. (A') Magnified view of GMR>GFP expression domain. (B, B') Misexpression of Aß42 (GMR>Aß42) restricts the Aß42 expression (green channel) to the differentiating photoreceptor neurons as detected using 6E10 antibody (red channel) in eye imaginal disc. (C, C') Normal eye development in (C) third instar larval eye imaginal disc and (C') third instar larval eye imaginal disc. (E, E') early pupal (white pupa) retina. (D, F) Misexpression of Aß42 (GMR>Aß42) results in the induction of cellular phenotypes in the (D) developing third instar larval eye imaginal disc and (F) early pupal retina. Disc large (Dlg, red channel) marks the membrane, Elav (green channel), a proneural marker that marks the neuronal fate. (D, D') In the third instar larval stage, morphological changes are not as severe but exhibit slight "holes" (arrows) as a result of Aß42 plaque accumulation. (F) In early pupal retina, there is an increase in disarray throughout the eye field. (F') The ommatidial clusters are clumped or fused together (arrows, outlines of ommatidial clumps marked by dotted line), (E) unlike the wild-type ommatidia.

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Figure 2. The pupal retina is a highly organized structure comprising of mature ommatidia (Figure 2A, C). GMR-Ga4 drives expression of GFP reporter (Figure 2A, A') and Aß42 in all the cell types of the pupal retina (Figure 2B, B'). The wild-type pupal retina shows a normal array of ommatidial clusters (Figure 2C, C') that are arranged in a hexagonal lattice whereas the pupal retina with Aß42 misexpression exhibits progressively severe phenotypes as compared to that seen in the early pupal retina (Figure 2C, C'). In GMR>Aß42 pupal retina, ommatidia do not retain the regular spacing due to multiple fusions, more of the ommatidial clusters are extruded and lost from the disc causing holes in the retina (Figure 2D, D', holes marked by red dotted line and arrow). The GMR>Aß42 mature retina (Figure 2F) is smaller than the wild-type control (Figure 2E), suggesting that the retina undergoes considerable cell loss during prepupal stages. This small pupal retina results in a small adult eye with glazed appearance and fusion of ommatidia (Figure 2F) as compared to the wild type adult eye (Figure 2E). In the adult eye with Aß42 misexpression (GMR>Aß42), a range of neurodegenerative phenotypes were observed. We found that (~55%) percent of the flies showed mild fusion of the ommatidia with reduced eye size (data not shown) where as the stronger phenotypes resulted in glazed appearance (~40%) of the adult eye along with the near complete fusion of the ommatidia of the compound eye (Figure 2F). A vertical section of the wild-type adult eye shows the separation of the lenses and the depth of the photoreceptors that constitute the retina (Figure 2G). However, the adult eye sections of GMR>Aß42 indicate that the retina is very thin, has poorly differentiated photoreceptors, the lenses are fused with the underlying retina and has a vacuolated morphology (Figure 2H, arrow). Overall, these studies indicate that Aß42 induces an early pathology in photoreceptors that continues to deteriorate as the retina ages and differentiates, leading to a small and very disorganized adult eye. The gaps in early pupal retina and the small adult eye suggest that cell death may play a prominent role in Aß42 neurotoxicity in the eye. It is important to investigate how early these cells (GMR>Aß42) begin to die after Aß42 misexpression.

Misexpression of Aß42 induces cell death in the developing eye

We performed TUNEL staining to investigate the timing of onset of cell death due to misexpression of Aß42 (GMR>Aß42) in early third instar larval eye imaginal disc. It is known that a few random cells undergo cell death in the wild-type third instar larval eye imaginal disc (Figure 3C, C') that does not affect the final morphology of the adult compound eye (Figure 3B). We found that TUNEL positive cells, which mark the nuclei of the dying cells, begin to appear as early as late third instar eye imaginal disc (Figure 3C, C'). The number of dying cells in the GMR>Aß42 larval eye imaginal disc was significantly higher compared to the wild-type eye imaginal disc (Figure 3I). In comparison to the wild-type adult eye (Figure 3B) the Aß42 misexpression (GMR>Aß42) leads to a strong neurodegenerative phenotype of near complete loss of the adult eye (Figure 3D). In order to test whether cell death is due to induction of the intrinsic caspase dependent cell death pathway, we misexpressed baculovirus P35 along with Aß42 (GMR>Aß42+P35), and found that it resulted in partial rescue of...
Role of JNK Signaling in Neurodegeneration

cell death in the third instar larval eye imaginal disc. The GMR>Aß42+P35 eye imaginal disc exhibit a significant reduction in number of dying cells (Figure 3E, E', 3I) and develop into adult flies that show subtle rescue of the adult eye field as the neurodegenerative phenotype was still present (Figure 3F). Thus, even though blocking caspase dependent cell death showed significant rescue in the larval eye imaginal disc, the adult eye showed a relatively stronger neurodegenerative phenotype suggesting that the protective role of blocking caspase dependent cell death in GMR>Aß42 is restricted to the early larval stages of eye development. Since blocking the caspases did not completely rescue the small and disorganized adult eye therefore, we tested the role of JNK pathway, a caspase-independent cell death pathway, in Aß42 neurotoxicity. To inhibit the JNK pathway, we misexpressed Puckered (Puc), a dual phosphatase that negatively regulates JNK [20,29]. Misexpression of puc in GMR>Aß42 background (GMR>Aß42+puc) showed a significant rescue of the cell death in the eye imaginal disc (Figure 3G, G') that resulted in a strong rescue of neurodegenerative phenotype in the adult eye (Figure 3H). Although the adult eyes have slightly disorganized ommatidia (Figure 3H, I), the extent of rescue was significantly higher than the GMR>Aß42+P35 adult eyes (Figure 3F, I). Our results suggest that although both caspase dependent as well as caspase-independent cell death through activation of JNK signaling pathway play an important role in Aß42 neurotoxicity in the Drosophila eye, the effects of JNK signaling was more prominent.

**Aß42 activates JNK signaling in the eye**

We tested if JNK signaling pathway is activated upon accumulation of Aß42 in the eye. We analyzed the expression of *puc*, a downstream target of JNK signaling pathway (Figure 4A). Since *puc* gene is a transcriptional target of JNK signaling, the expression of *puc-lacZ* reporter serves as a functional read-out of JNK activity [43]. In the control eye imaginal disc, weak expression of *puc* enhancer trap line is detectable in photoreceptor precursors (Figure 4B, B'). However, in GMR>Aß42 eye imaginal disc, we observed strong induction of *puc-lacZ* expression (Figure 4C), especially in the most posterior domain that has expressed Aß42 longer (Figure 3G, G'). This data suggests that JNK signaling is activated in GMR>Aß42 eye imaginal disc. To confirm these results, we quantified the amount of phospho-Jun (p-Jun) present in GMR>Aß42 eye imaginal disc cells. Jun kinase (JNK) is known to encode an enzyme that can phosphorylate N-terminal of its substrate Jun [49]. The phospho-Jun quantification can provide the activation status of JNK signaling pathway. We found that in GMR-Gal4>Aß42 eye imaginal disc cells, the p-Jun levels are three times higher than the wild-type eye imaginal disc (Figure 4D). Together, this data suggests that JNK signaling is rapidly activated by Aß42 in the eye imaginal disc.

**Aß42 mediated neurodegeneration in the eye is due to activation of JNK signaling**

We investigated the role of JNK signaling in Aß42 misexpression mediated neurotoxicity by modulating the activity of components of the JNK pathway. We found that in GMR>Aß42 background, the strong induction of puc-lacZ reporter in the eye imaginal disc is accompanied by dramatic increase in frequency of dying cells (TUNEL positive cells; Figure 3B) as compared to the wild-type eye (Figure 3A). Furthermore, Aß42 misexpression (GMR>Aß42) result in a strong neurodegenerative phenotype in...
Figure 3. Misexpression of Aβ42 triggers cell death in the differentiating neurons. (A, A’) Wild-type third instar larval eye imaginal disc displaying randomly distributed TUNEL positive dying cells (A, A’) shown in red channel (arrow). TUNEL staining marks the fragmented DNA within the nuclei of dying cells [29,32,61]. (B) Wild-type adult eye. (C, C’) Misexpression of Aβ42 (GMR>Aβ42) in differentiating neurons of the eye show elevated levels of TUNEL positive cells (C’ arrows). The increased frequency of cell death in neurons can be directly correlated to the misexpression of the Aβ42 peptide. Note that misexpression of Aβ42 does not affect the differentiation process as the distribution of Elav positive cells is the same in both control and Aβ42 third instar eye imaginal discs. (D) GMR>Aβ42 results in a strong neurodegenerative phenotype in adult eye. Baculovirus P35 has been shown to block the caspase dependent cell death [36]. (E, E’, F) Misexpression of P35 along with Aβ42 in differentiating neurons (GMR>Aβ42+P35) shows significant reduction of dying cells based on number of TUNEL positive cells (red channel) in the larval eye field. However, this rescue is not as strong in (F) adult eye phenotype. (E’) Note that the eye field displays reduced number of TUNEL positive cells (arrow) compared to GMR>Aβ42 eye field (C’). It is important to note that Aβ42 peptide production is not affected. Elav marks the photoreceptor fate (C’’). Puckered (Puc), a dual phosphatase, is downstream target of JNK signaling pathway and forms a feedback loop to negatively regulate the pathway [20]. (G, G’, H) Misexpression of puc along with Aβ42 in the differentiating neurons (GMR>Aβ42+puc) results in significant suppression of cell death as evident from reduced number of TUNEL positive cells in the third instar larval eye imaginal disc as well as in the (H) adult eye. Note that there is a significant rescue of (D) GMR>Aβ42 adult eye phenotype in (H) GMR>Aβ42+puc background. These results suggest that JNK signaling might be responsible for neurodegeneration seen in amyloid plaque mediated cell death. (I) Quantification of the number of dying cells in eye imaginal discs based on TUNEL.
staining in wild-type (served as control), GMR->Aβ42, GMR->Aβ42+P35 and GMR->Aβ42+puc. Note that blocking JNK signaling (GMR->Aβ42+puc) exhibit strong rescue of the neurodegenerative phenotype of GMR->Aβ42 and GMR->Aβ42+P35. This rescue is significant (***) as seen by calculation of P-values based on one-tailed t-test using Microsoft Excel 2007. doi:10.1371/journal.pone.0024361.g003

the adult eye (Figure 5D) as compared to the wild-type adult eye (Figure 5C). Thus, if JNK signaling is involved in neurodegeneration in GMR->Aβ42 background, then reducing JNK signaling levels would rescue the phenotype whereas increasing the levels of JNK signaling will have converse effect. We used several components of JNK signaling pathway (Figure 4A) to address our hypothesis and analyzed the eye phenotypes at the eye imaginal disc levels as well as the adult eye.

To activate JNK signaling, we expressed constitutively active hemipterous (hep*) and Djun (junaspv7). We found that misexpression of constitutively active hemipterous, GMR->Aβ42+hep (Figure 5F) or constitutively active Djun, GMR->Aβ42+junaspv7 (Figure 5J) enhances the frequency of TUNEL positive (dying) cells in the eye imaginal disc in comparison to their respective controls viz., GMR->hep (Figure 5E) and GMR->junaspv7 (Figure 5I). Similar phenotypes were observed in the adult eyes of GMR->Aβ42+hep (Figure 5G) and GMR->Aβ42+junaspv7 (Figure 5K). Not surprisingly, GMR->hep* (Figure 5F) as compared to the wild-type eye imaginal disc levels as well as the adult eye

induce the aberrant development of the adult eye field, due to the increase in cell death. However, the phenotypes of only activation of JNK signaling pathway in eye as seen in controls are much weaker (Figure 5E, I, G, K) then when JNK signaling pathway is activated in GMR->Aβ42 background (Figure 5 F, H, J, L). Thus, activation of JNK signaling significantly increases the induction of cell death by Aβ42, supporting the potential involvement of JNK in Aβ42 neurotoxicity.

We further tested this hypothesis by analyzing the effect of reducing the levels of JNK signaling on GMR->Aβ42 neurodegenerative phenotype by using a dominant negative Basket allele (bskDN). GMR->bskDN, which serve as a control, does not affect developmental cell death in the eye imaginal disc (Figure 5M) and the adult eye (Figure 5O). However, misexpression of bskDN along with Aβ42 (GMR->Aβ42+bskDN) dramatically reduces the number of apoptotic cells in the eye imaginal disc (Figure 5N). This protective effect of bskDN (GMR->Aβ42+bskDN) further continued during pupal and adult eye development, resulting in

Figure 4. JNK signaling is activated upon misexpression of Aβ42 in the eye. (A) Schematic representation of hierarchy of Jun-kinase signaling pathway members. (B, B’’) Wild-type expression of puc in the developing third instar larval eye imaginal disc using lacZ reporter where reporter (red channel) is restricted only to the developing photoreceptors in the eye imaginal disc proper and in the peripodial membrane cells on the margin of the antennal disc [43]. (C, C’) GMR->Aβ42 eye imaginal disc exhibits ectopic upregulation of puc-lacZ reporter. (C’) Split channel showing ectopic puc-lacZ expression in the photoreceptor neurons of the eye imaginal disc. (D) Activation of JNK signaling in GMR->Aβ42 was detected by checking phospho-Jun levels. Levels of JNK signaling pathway increases three fold in GMR->Aβ42 as compared to the wild-type eye imaginal disc.

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a significantly rescued, larger eye in the adult fly that still showed some ommatidial disorganization (Figure 5P). These results are consistent with the protective effect of Puc overexpression as shown in Figure 3. Overall, these studies demonstrate that JNK signaling regulates Aβ42-induced cell death in the eye.

JNK signaling plays major role in Aβ42-plaque mediated cell death

Since blocking either JNK signaling or caspase-dependent cell death alone does not result in complete rescue of the eye phenotype induced by Aβ42 (Figure 3, 5), we next tested whether...
blocking both JNK signaling and caspase-dependent cell death pathways at the same time provided additional protection. Misexpression of both P35 and puc together in GMR>Aß42 background (GMR>Aß42+P35+puc) results in highly reduced cell death in the eye imaginal disc (Figure 6A, A', E), even though robust levels of Aß42 are induced. However, the extent of cell death based on number of dying cells in eye imaginal disc was not significantly different from GMR>Aß42+puc (Figure 3E, 6E) and GMR>Aß42+P35 (Figure 3G, 6E). The pupal retina of GMR>Aß42+P35+puc also showed similar phenotype of highly reduced cell death.

**Figure 6. JNK signaling is responsible for cell death in GMR>Aß42 background.** (A, A') Misexpression of both P35 and puc along with Aß42 (GMR>Aß42+P35+puc) results in strong rescue of cell death as evident from (A') dramatically reduced TUNEL positive cells. However, the rescue of the phenotype was not significantly stronger than with blocking JNK signaling pathway alone (Figure 5G'). (B) Misexpression of Aß42 (GMR>Aß42) in pupal retina showing cell death as evident from TUNEL staining (red channel). Blocking simultaneously both caspase-dependent cell death and caspase-independent JNK signaling mediated cell death in pupal retina (GMR>Aß42+P35+puc) showed a strong rescue in (C, C', C'') pupal retina and (D) adult eye as compared to (B) GMR>Aß42 pupal retina, (Figure 3D) GMR>Aß42 adult eye. The cell death is detected by TUNEL staining (red channel), which is (C', C) restricted to the periphery of the pupal retina. Note that dying cells on the periphery of the pupal retina corresponds to the programmed cell death as seen in the wild-type pupal retina too [25,26]. (E) Quantification of the number of dying cells in eye imaginal discs based on TUNEL staining in different genetic combinations. The frequency of cell death in wild-type eye imaginal disc served as a control. Note that blocking JNK signaling (GMR>Aß42+puc) or blocking JNK signaling along with caspase–dependent cell death (GMR>Aß42+P35+puc) exhibit strong rescue of the neurodegenerative phenotype of GMR>Aß42. This rescue is significant (**) as seen by calculation of P-values based on one-tailed t-test using Microsoft Excel 2007. doi:10.1371/journal.pone.0024361.e006
cell death (Figure 6C) as compared to GMR->Aß42 pupal retina which shows significant number of TUNEL positive dying cells (Figure 6B). At this time the pupal retina shows very little cell death, which is actually restricted only to the periphery of the retina where Wg signaling eliminates the extra retinal precursor cells by PCD [20] that limits the eye/head capsule boundary (Figure 6C). Blocking both cell death pathways (GMR->Aß42+P35+pac) showed a significant rescue in the adult eye (Figure 6D). However, the rescue was comparable to the one seen by blocking JNK signaling pathway alone (Figure 3H, 5M). Thus, overall the rescue by blocking JNK signaling alone (GMR->Aß42+BskDN, Figure 5K, M) was comparable to the one seen by blocking both P35+JNK signaling (GMR->Aß42+P35+pac; Figure 6D). Thus, our results strongly suggest that JNK signaling pathway is activated during Aß42-plaque mediated neurodegeneration in the fly retina.

Discussion

One of the characteristic features of neurodegenerative disorders like AD and Parkinson disease (PD) is the late onset of neuropathology due to aberrant cellular homeostasis probably due to misregulation of several signaling pathways involved in growth, patterning and survival [2,4,5,50]. Thus, it is apparent that these neurodegenerative disorders are not due to a single gene mutation but a cumulative outcome of impairment of a large spectrum of signaling pathways. Therefore, in order to understand the complexity of the human disorders and to develop therapeutic approaches, it is important to discern the role of various signaling pathways in the neuropathology caused by Aß42-plaques. This evident complexity is one of the reasons why neurodegenerative diseases are so difficult to understand and treat. Our goal here was to tease out the role of the cell death pathways in Aß42 neurotoxicity. It has been known for some time that high levels of Aß42 result in small and disorganized phenotypes of eyes that contain thin retinas with poorly differentiated photoreceptors [12,14,45]. This small eye suggests that Aß42 induces extensive cell death in the developing eye. To understand when the cell death occurs, we studied how the maturation of photoreceptors is affected by the presence of Aß42.

We have employed the highly versatile model of Drosophila eye to understand the role of signaling pathways involved in cell death in Aß42-plaque mediated neuropathology [3,10,11,14]. Since the eye is dispensable for the survival of fly, the transgenic Drosophila eye model is ideal for these studies as we can assay the effects throughout eye development without killing the fly. Our data suggest that neurodegeneration in the fly retina can be triggered as early as third instar eye imaginal disc using GMR-Gal4 driver mediated misexpression of Aß42 (GMR->Aß42; Figure 1, 3), which is only a few hours after Aß42 expression starts in the developing eye field. We also found that even though cell death is induced as early as the third instar eye imaginal disc, the morphology of the developing eye field does not dramatically differ between the wild type eye versus the GMR->Aß42. At this time the toxicity of Aß42 is only apparent at the level of cell membranes, which shows minor effects on cell arrangement (Figure 1D, D'). However, the number of the dying cells shows dramatic increase in GMR->Aß42 eye imaginal disc as compared to the wild-type eye imaginal disc (Figure 1, 3). Thus, genetic programming that triggers the onset of Aß42-plaque mediated neurodegeneration is activated soon after the onset of misexpression of Aß42 in the developing retina. Therefore, the experiments to demonstrate rescue of neurodegeneration phenotype should take this time window into consideration.

The larval eye imaginal disc metamorphose into the prepupal retina, which shows clumping of photoreceptor clusters, an indication that photoreceptor specification and signaling are aberrant (Figure 1F, F'). The clumping phenotype is caused by fusion of photoreceptor neurons and results in loss of ommatidial cluster integrity. Despite these changes at the photoreceptor neurons level, the outline of the pupal retina shows subtle effects (Figure 1). In the late pupal retina, the size of the retina begins to reduce as the severity of the phenotypes increases at this stage. In the late pupal stage, the retina contains holes due to loss of photoreceptors. The outcome of this cellular aberrations in the eye leads to a small adult eye with glazed appearance and fused ommatidia. Thus, extensive cell death is responsible for some of the phenotypes observed in the adult eye expressing Aß42. Not surprisingly, the neurodegenerative phenotypes exhibited by Aß42-plaque are age and dose dependent. Since the Gal4-UAS system is temperature sensitive, it serves as an excellent source to test the dose dependence [51,52]. The cultures reared at 25°C showed less severe phenotypes as compared to the ones reared at 29°C (data not shown). Furthermore, the severity of phenotypes increased with the age (Figure 2).

The next plausible question was, which pathways mediate the extensive cell death induced by Aß42? Our idea was to test the caspase-dependent pathway since the majority of cell death is triggered by activation of caspase-dependent cell death in tissues. To demonstrate the role of caspases in Aß42-mediated cell death, we show that the misexpression of baculovirus P35 protein [36], significantly reduce the number of TUNEL-positive cells in the larval eye disc (Figure 6E). Interestingly, unlike the larval eye disc, the adult eyes did not show comparable strong rescues. It seems there is block in cell death mainly during the larval eye imaginal disc development but the adult eye exhibits a weaker rescue of GMR->Aß42 neurodegenerative phenotype. This reduction in cell death supports the possible role of caspase-mediated cell death in the small eye induced by Aß42. However, the eye of GMR->Aß42+P35 is reduced and disorganized (partial rescue), suggesting that other pathways contribute to Aß42 neurotoxicity in the eye.

Aß42 neurotoxicity is mediated through activation of JNK signaling pathway

JNK-mediated caspase-independent cell death also plays an important role in tissue homeostasis during development. JNK signaling, a family of multifunctional signaling molecules, is activated in response to a range of cellular stress signals and is a potent inducer of cell death [20]. Consistent with this, Aß42 activates JNK signaling in the eye imaginal disc as indicated by the transcriptional regulation of puc and Jun phosphorylation (Figure 4). Moreover, JNK signaling upregulation increases cell death, supporting the role of JNK in Aß42 neurotoxicity (Figure 5). Conversely, blocking JNK signaling dramatically reduces cell death in larval eye imaginal disc (Figure 6E) and the resulting flies from blocking JNK signaling exhibit large and well organized eyes (Figure 5). Thus, we were able to identify the JNK signaling pathway as a major contributor to cell death observed in the Aß42 eyes. Our studies also highlight that cell death response to misexpression of Aß42-plaques is way earlier before its affect can be discernible at the morphological level. Since neurons are post-mitotic cells, they can not be replaced. Therefore, early detection of the onset of neurodegeneration is crucial. If the disease is detected later, it may only be possible to block the further loss of healthy neurons. However, the neurons lost prior to block of cell death will not be replaced. It is possible that JNK signaling activation may serve as an early bio-marker for Aß42 plaque mediated neuropathology. Thus, members of JNK signaling pathway can serve as excellent biomarkers or targets for the therapeutic approaches.
We found that blocking JNK signaling significantly rescued the neurodegenerative phenotypes but the eyes still show subtle signs of Ab42 in the disorganization of the lattice. Therefore, we blocked both caspase dependent cell death and JNK signaling in fly retina misexpressing Ab42. Blocking both caspase and JNK pathways simultaneously produced the protection against Ab42, suggesting that Ab42 induces cell death by several mechanisms. Our results suggest that blocking multiple pathways may result in significant protection against Ab42 neurotoxicity, an important consideration for potential AD therapies.

**JNK signaling pathway plays role in cell survival**

JNK signaling pathway has been known to be involved in different processes of ageing and development, including tissue homeostasis, cell proliferation, cell survival and innate immune response. Interestingly, evidence collected in several models of AD supports the involvement of JNK signaling in AD. Consistent with our observations, Ab42 induces JNK activation in primary cultures of rat cortical neurons [53]. Also, the kinase activity of JNK phosphorylates Tau in vitro, thus contributing to the production of hyperphosphorylated Tau, one of the key toxic molecules in AD [54]. Moreover, inhibition of JNK with peptides prevented cell loss in an Tg2576; PS1M146L brain slice model [18]. Additionally, it has been shown that the neuroprotective effect of the diabetes drug rosiglitazone inhibits JNK and results in reduced Tau phosphorylation in rat and mice [55]. Our results support these findings in mammalian models of AD, and provide the first evidence that direct manipulation of JNK activity modulates Ab42 neurotoxicity in vivo. Despite this evidence, JNK is currently not a major pathway in AD research. Our results, together with the published literature, suggest that more attention should be paid to the role of JNK in AD pathogenesis and its potential as a therapeutic target and biomarker.

In fact, the protective activity of JNK may not be limited to AD, as JNK inhibition may show beneficial effects in other diseases, including PD, stroke and others [49].

**Materials and Methods**

**Stocks**

Fly stocks used in this study are described in Flybase (http://flybase.bio.indiana.edu). Stocks used include: GMR-Gal4 UAS-Ab42, and puc [50], a lacZ reporter which expresses under the control of puc endogenous regulatory elements acts as a functional readout of JNK signaling pathway [43].

We have used Gal4/UAS system for targeted misexpression studies [56]. All Gal4/UAS crosses were done at 18°C, 25°C and 29°C, unless specified, to sample different induction levels. All the experiments were conducted using the Glass Multimer Reporter driver line (GMR-Gal4), which directs expression of transgenes in the differentiating retinal precursor cells of the developing eye [46]. The responder strains were: UAS-Ab42 [14,45], UAS-bik [37], UAS-DJNK1 [57], UAS-puc [43], UAS-p35 [36], P{UAS-GFP.S65T/T10} [58].

**Immunohistochemistry**

Eye-antennal imaginal discs were dissected from wandering third instar larvae and stained following the standard protocol [59]. Pupal retina were dissected and stained with combinations of antibodies using the standard protocol [59,60]. Antibodies used were mouse and rabbit anti-ß-galactosidase (1:200) (Cappel); rat anti-ß-galactosidase (1:200); mouse anti-Wg (1:50) (Developmental Studies Hybridoma Bank; mouse anti-Dlg (a gift from K. Cho). Secondary antibodies (Jackson Laboratories) were goat anti-rat IgG conjugated to Cy5 (1:200), donkey anti-rabbit IgG conjugated to Cy3 (1:250), donkey anti-rabbit IgG conjugated to FITC, donkey anti-mouse IgG conjugated to Cy3 (1:200). Immunofluorescent images were analyzed using the Olympus Fluoview 1000 Laser Scanning Confocal Microscope. All final figures were prepared using Adobe Photoshop software.

**Detection of Cell Death**

Apoptosis was detected by using TUNEL assays [29,32,61]. TUNEL assays are used to identify cells undergoing apoptosis where the cleavage of double and single stranded DNA is marked effectively. This protocol involves labeling DNA breakage by adding fluorescently labeled nucleotides to free 3’-OH DNA ends in a template-independent manner using Terminal deoxynucleotidyl transferase (TdT). The fluorescent labels incorporated in nucleotide polymers can be detected by fluorescence microscopy. Eye-antennal discs, after secondary-antibody staining [59], were blocked in 10% normal goat serum in phosphate buffered saline with 0.2% Triton X-100 (PBT) and labeled for TUNEL assays using a cell-death detection kit from Roche Diagnostics. The TUNEL positive cells were counted from five sets of imaginal discs and were used for the statistical analysis using Microsoft Excel 2007. The P-values were calculated using one-tailed t-test, and the error bars represent Standard Deviation from Mean.

**Histology**

For histological analysis of retinas, epon-embedded heads of one day-old flies were sectioned at 1 µm and stained with toluidine-blue as described before [62]. Sections were documented in a Nikon 80i microscope with a Zeiss AxioCam digital camera and AxioVision software.

**Western Blot**

Protein samples were prepared from eye-antennal imaginal discs from third instar wild type and GMR->Ab42 larvae in PBS and then subjected to boiling in Laemli’s sample buffer containing SDS-6 mercaptoethanol for 10 minutes. Samples were resolved on a 10% gel, and transferred on to nitrocellulose membrane. The blot was washed with 1x TBST (pH 7.5) for 10 min each (X3); incubated in 5% w/v BSA in 1x TBST (pH 7.5) overnight. The blot was recovered from blocking solution the following day, and incubated in diluted 1:1000 Phospho-SAPK/JNK (Cell Signalling Thr183/Tyr185) (81E11) Rabbit antibody diluted in 5% w/v BSA in 1x TBST at 4°C with gentle shaking overnight. Signal was detected using horseradish peroxidase-conjugated goat anti-rabbit IgG using super signal chemiluminescence substrate (Pierce). Images were captured using the BioSpectrum® 500 Imaging System.

**Scanning Electron Microscopy (SEM)**

The flies were prepared for scanning electron microscopy through a series of increasing concentrations of acetone [63]. Dehydrated flies were then incubated in 1:1 acetone and HMDS (Hexa Methyl Di Silazane, Electron Microscopy Sciences) for 24 hrs followed by incubation in 100% HMDS. The flies were allowed to air dry in HMDS in the hood. Dehydrated flies were mounted on Electron microscopy stubs. Flies were coated with gold using a Denton vacuum sputter coater and analyzed using a Hitachi S-4800 High Resolution Scanning Electron Microscope (HRSEM).

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Author Contributions
Conceived and designed the experiments: AS MK-S. Performed the experiments: MT RM JN ORP SB. Analyzed the data: AS MK-S PF. Contributed reagents/materials/analysis tools: PF-F MT MK-S AS. Wrote the paper: AS MK-S PF-F

References

Author Contributions
Conceived and designed the experiments: AS MK-S. Performed the experiments: MT RM JN ORP SB. Analyzed the data: AS MK-S PF. Contributed reagents/materials/analysis tools: PF-F MT MK-S AS. Wrote the paper: AS MK-S PF-F

References

Hybridoma Bank (DSHB) for the antibodies; and members of the Singh and Kang-Singh lab for critical comments on the manuscript.

Role of JNK Signaling in Neurodegeneration

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Conceived and designed the experiments: AS MK-S. Performed the experiments: MT RM JN ORP SB. Analyzed the data: AS MK-S PF. Contributed reagents/materials/analysis tools: PF-F MT MK-S AS. Wrote the paper: AS MK-S PF-F

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Dorsal eye selector pannier (pnr) suppresses the eye fate to define dorsal margin of the Drosophila eye

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ABSTRACT

Axial patterning is crucial for organogenesis. During Drosophila eye development, dorso-ventral (DV) axis determination is the first lineage restriction event. The eye primordium begins with a default ventral fate, on which the dorsal eye fate is established by expression of the GATA-1 transcription factor pannier (pnr). Earlier, it was suggested that loss of pnr function induces enlargement in the dorsal eye due to ectopic equator formation. Interestingly, we found that in addition to regulating DV patterning, pnr suppresses the eye fate by downregulating the core retinal determination genes eyes absent (eya), sine oculis (so) and dachshund (dac) to define the dorsal eye margin. We found that pnr acts downstream of Ey and affects the retinal determination pathway by suppressing ey. Further analysis of the “eye suppression” function of pnr revealed that this function is likely mediated through suppression of the homeotic gene teashirt (tsh) and is independent of homothorax (hth), a negative regulator of eye. Pnr expression is restricted to the peripodial membrane on the dorsal eye margin, which gives rise to head structures around the eye, and pnr is not expressed in the eye disc proper that forms the retina. Thus, pnr has dual function, during early developmental stages pnr is involved in axial patterning whereas later it promotes the head specific fate. These studies will help in understanding the developmental regulation of boundary formation of the eye field on the dorsal eye margin.

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Introduction

Axial patterning is required for the transition of a single sheet of cells into a three-dimensional organ. Axial patterning involves generation of two different cell populations or compartments. Compartments are the fundamental units of patterning that are generated by localized expression of transcription factors called selectors. The selectors, when expressed in a group of cells, can confer compartment-specific properties to these cells (Curtiss et al., 2002; Mann and Carroll, 2002). Signaling between the cells of two compartments is crucial for the patterning, growth and differentiation of a developing field (Blair, 2001). The developing eye of the fruit fly, Drosophila melanogaster, has been extensively used to study patterning and growth. The compound eye of the adult fly develops from an epithelial bi-layer called the eye-antennal imaginal disc that is derived from the embryonic ectoderm (reviewed by Cohen, 1993, Held, 2002). The imaginal disc is a sac-like structure present inside the larva, which is the product of two different layers: the peripodial membrane (PM) and the disc proper (DP). The Drosophila retina develops from the DP while the PM of the eye-antennal imaginal disc contributes to the adult head structures (Milner et al., 1983; Haynie and Bryant, 1986; Atkins and Mardon, 2009). Morphogenesis during animal development involves signaling between different layers of the tissue (Furuta and Hogan, 1998; Obara-Ishihara et al., 1999). Similarly, signaling between the PM and the DP of the eye disc is essential for dorso-ventral (DV) axis establishment and patterning (Cho et al., 2000; Gibson and Scubiger, 2000; Atkins and Mardon, 2009).

The adult eye is a highly precise hexagonal array of ~800 ommatidial clusters or unit eyes (Ready et al., 1976; Wolff and Ready, 1993). The ommatidia are arranged in two chiral forms, which are in a mirror image asymmetry along the DV midline called the equator. The equator demarcates the boundary between the dorsal and the ventral eye, and is the site for upregulation of Notch (N) signaling, which triggers cell proliferation and differentiation (Cho and Choi, 1998; Domínguez and de Celis, 1998; Papayannopoulos et al., 1998; Singh et al., 2005b). Although the mirror image asymmetry is generated during the third instar stage of larval eye development, the subdivision of the eye into dorsal and ventral compartments takes place even earlier by domain specific expression and function of DV patterning genes (Cho and Choi, 1998; Domínguez and de Celis, 1998; Papayannopoulos et al., 1998; Cavodeassi et al., 2005).
1999; Maurel-Zaffran and Treisman, 2000; Singh et al., 2005b). In antenna, wing and leg imaginal discs, DV boundary formation takes place after the antero-posterior (AP) lineage restriction is generated (Blair, 2001; Garcia-Bellido and Santamaria, 1972; Mílan and Cohen, 2003; Morata and Lawrence, 1975; Tabata et al., 1995). However, in the eye disc, the AP pattern is established dynamically in the third larval instar stage (after DV lineage is established) when the morphogenetic furrow (MF) is initiated (Singh et al., 2005b). The MF is a wave of retinal differentiation, which progresses anteriorly resulting in the transformation of undifferentiated retinal precursor cells (anterior to MF) into differentiated photoreceptor neurons (posterior to MF) of the eye (Ready et al., 1976; Wolff and Ready, 1993; Heberlein and Moses, 1995; Lee and Treisman, 2001a,b).

Therefore, the DV lineage, which is established at late first instar or early second instar larval stages, is the first lineage restriction event in the eye, and is crucial for the growth and differentiation of the eye (Singh and Choi, 2003; Singh et al., 2005b).

During genesis of eye, the entire early eye imaginal primordium initiates from the default ventral fate, which depends on the functions of Lobe (L) and Serrate (Ser) genes (Chern and Choi, 2002; Singh and Choi, 2003; Singh et al., 2005a,b, 2006). The onset of expression of the dorsal selector gene pnr, a member of the GATA-1 family of transcription factors, at the dorsal margin of early second instar larval eye discs establishes the DV lineage in the eye (Maurel-Zaffran and Treisman, 2000; Singh and Choi, 2003; Singh et al., 2005a). It has been shown that pnr (Maurel-Zaffran and Treisman, 2000) and members of the Iro-C homeodomain genes viz., araucan (ara), caupolican (caup) (Cavodeassi et al., 1999) and mirror (mirr) (Kehl et al., 1998; McNeil et al., 1997) are expressed in the dorsal region of the prospective eye (Domínguez and de Celis, 1998; McNeil et al., 1997) and act as the dorsal eye fate selectors. pnr, the most upstream gene known in the dorsal eye gene hierarchy, regulates the expression of downstream Iro-C genes through Wingless (Wg) signaling (Heberlein et al., 1998; Maurel-Zaffran and Treisman, 2000). Wg, which encodes a secreted protein, is expressed along the antero-lateral margins of the third instar eye-antennal imaginal disc (Baker, 1988), and prevents ectopic initiation of retinal differentiation from these positions (Ma and Moses, 1995; Treisman and Rubin, 1995). Wg signaling promotes the growth of cells in the eye-antennal disc and is sufficient to maintain cells in an undifferentiated state such that these cells continue to express anterior head specific markers (Lee and Treisman, 2001a,b). In the dorsal eye, Wg promotes expression of Iro-C genes during early eye development. The dorsal eye genes and the genes involved in ventral eye development act antagonistically to each other (Singh et al., 2005a,b). These genetic interactions define a signaling pathway that contributes toward the positioning of the equator (Cho and Choi, 1998; Domínguez and de Celis, 1998; Papayannopoulos et al., 1998; Maurel-Zaffran and Treisman, 2000). Thus, pnr is known to specify dorsal eye fate. However, the role of pnr during retinal differentiation of the eye is not known. Therefore, it is important to discern the role of DV patterning gene, pnr, during later stages of eye development.

Interestingly, loss-of-function of DV patterning genes manifest defects in the eye growth and patterning but the mechanism by which the DV patterning genes contribute to retinal determination is unknown. It is known that eye specific, and determination depends on a core of retinal determination (hereafter, RD) genes. These RD genes include PAX-6 homolog eyeless (ey), twin of eyeless (toy), eyes absent (eya), sine oculis (so) dachshund (dac), optix (opr), and eye gene (eyg) (Pappu and Mardon, 2004; Domínguez and Casares, 2005; Kumar, 2009). Ey is one of the early expressed genes which is required for eye field specification and is reported to induce expression of eya and so to promote eye growth and cell fate specification (for review Pappu and Mardon, 2004; Silver and Rebay, 2005; Kumar, 2009). The multiple feedback and cross regulatory interactions among RD genes lead to formation of the eye. Loss-of-function of RD genes results in the loss of eye field whereas ectopic expression of RD genes results in the induction of ectopic eyes (Haldet al., 1995; Pappu and Mardon, 2004; Silver and Rebay, 2005; Kumar, 2009). Several genes other than RD genes contribute towards eye development. A homeotic gene, tsh, which encodes a C-H2 Zinc finger transcription factor with three widely spaced Zinc finger domains (Fasano et al., 1991), has been suggested to act upstream of eya, so and dac during eye development (Pan and Rubin, 1998; Kumar, 2009).

Interestingly, tsh also exhibits asymmetric DV response in the eye (Singh et al., 2002). Misexpression of tsh suppresses the eye fate in the ventral eye and promotes ectopic dorsal eye enlargement (Singh et al., 2002). It has been shown that tsh collaborates with the genes that express in a domain specific manner to exhibit DV asymmetric response in the developing eye disc (Singh et al., 2004). Interestingly, in the dorsal eye, the gain-of-function phenotype of teashirt (tsh) is similar to the loss-of-function phenotype of pnr. However, the mechanism of their interaction during eye development is not fully understood. In the ventral eye, tsh suppresses the eye by induction of a Meis class of homeotic gene, homothorax (hth) (Riechhoff et al., 1997; Singh et al., 2002). Tsh has been shown to physically bind hth anterior to the eye field (Bessa et al., 2002). Hth, is known to act as a negative regulator of eye development. Loss-of-function of hth results in induction of the ventral eye or enlargement of the ventral eye domain (Pai et al., 1998). However, loss-of-function of hth in the dorsal eye has no effect even though hth is expressed in the dorsal eye (Pichaud and Casares, 2000; Jaw et al., 2000). Further, tsh does not affect hth expression in the dorsal eye whereas tsh acts upstream of hth in the ventral eye (Singh et al., 2002). Therefore, the mechanism of hth regulation in the dorsal eye remains unknown. Interestingly, the mechanism of genetic regulation of dorsal eye field growth is not very clear.

pnr plays an important role in the dorsal eye development. Onset of pnr expression in the dorsal eye margin is associated with DV lineage restriction (Maurel-Zaffran and Treisman, 2000; Cavodeassi et al., 2000; Singh et al., 2005b). The loss-of-function phenotypes of pnr result in ectopic eye enlargement of the dorsal eye. Here we report that in addition to its earlier reported role of dorsal selector during axial (DV) patterning, pnr plays an important role in defining the dorsal eye margin by regulating retinal determination. We have found that gain of function of pnr suppresses the retinal determination whereas the loss of pnr results in the ectopic induction of retinal determination genes. Our data suggests that pnr suppresses the retinal determination by downregulation of homeotic gene tsh, and is independent of hth. Interestingly, pnr is expressed only in the peripodial membrane and not in the disc proper, which gives rise to the retina. Thus, a late function of pnr is to block retinal determination in the peripodial membrane to define the dorsal eye margin.

Materials and methods

Stocks

Fly stocks used in this study are described in Flybase (http://flybase.bio.indiana.edu). We used y, w, eyFLP (Newsome et al., 2000), y; w; PFT82P pnrE943/Cyo, O (Heitzzler et al., 1996), UAS-pnrE24 (Haenlin et al., 1997), UAS-pnrE29 (Klinedinst and Bodmer, 2003), FRT82D hthP27 (Noro et al., 2006), UAS-ara (Gómez-Skarmeta and Modolell, 1998), UAS-hth12 (Pai et al., 1998), UAS-hthP358 (Inbal et al., 2001), y; tshF75CyO (Fasano et al., 1991); UAS-lishat (Gallet et al., 1998), UAS-ds tsh (Bessa and Casares, 2005), y; tshAD (Sun et al., 1995); y,w; UAS-NLS-GFP (Ito et al., 1997) UAS-wg (Azpiazu and Morata, 1998), pnr-Gal4, UAS-GFP (Singh et al., 2005a), ey-Gal4 (Hazelett et al., 1998), br-Gal4 (Calleja et al., 1996). We have used Gal4/UAS system for targeted misexpression studies (Brand and Perrimon, 1993). All Gal4/UAS crosses were done at 18 °C, 25 °C and 29 °C, unless specified, to sample different induction levels.
Genetic mosaic analysis

Loss-of-function clones were generated using the FLP/FRT system of mitotic recombination (Xu and Rubin, 1993). To generate loss-of-function clones of pnr in the eye, eyFLP; FRT82B Ubi-GFP females were crossed to y, w; FRT82B pnr[am] males. Gain-of-function clones of pnr were generated using hs-FLP method where y, w, hsFLP[22]; P(Act>γ+>Gal4) 25 P(UAS-GFP[26T])/CyO (Struhl and Basler, 1993; Ito et al., 1997) flies were crossed to UAS-pnr[24] flies.

Immunohistochemistry

Eye-antennal imaginal discs were dissected from wandering third instar larvae and stained following the standard protocol (Singh et al., 2002). Antibodies used were mouse and rabbit anti-β-galactosidase (1:200) (Cappel); chicken anti-GFP (1:200) (Upstate biotechnology), rat anti-Elav (1:100); mouse anti-Wg (1:50) (Developmental Studies Hybridoma Bank); rabbit anti-Dlg (a gift from K. Cho), rabbit anti-Ey (a gift from Uwe Walldorf and Patrick Callaerts), anti-Hth (a gift from H. Sun and R. Mann) mouse anti-So (1:100), mouse anti-Dac (1:100), mouse anti-Eya (1:100) (Developmental Studies Hybridoma Bank), Rat anti-Tsh (1:50) (Gallet et al., 1998). Secondary antibodies (Jackson Laboratories) were goat anti-rabbit IgG conjugated with Cy5 (1:200), donkey anti-rabbit IgG conjugated to Cy3 (1:250), donkey anti-mouse IgG conjugated to Cy3 (1:200). Pnr expression was detected using pnr-Gal4>UAS-GFP (Pichaud and Casares, 2000; Singh and Choi, 2003; Singh et al., 2005a). Immunofluorescent images were analyzed using the Olympus Fluoview 1000 Laser Scanning Confocal Microscope.

Scanning electron microscopy (SEM)

The flies were prepared for scanning electron microscopy by dehydration through a series of increasing concentrations of acetone. Dehydrated flies were then stored in 1:1 mixture of acetone and Hexamethyl Di Silazane (HMDS, Electron Microscopy Sciences), and then stored in 100% HMDS. The flies were allowed to dry in HMDS. Dehydrated flies were mounted on a carbon conductive tape on EM stubs. Fly samples were coated with gold using a Denton vacuum sputter coater and analyzed using a Hitachi S-4800 High Resolution Scanning Electron Microscope (HRSEM).

Results

Pnr expression is restricted to the peripodial membrane (PM) of the dorsal eye margin

In the Drosophila embryo, pnr is expressed in the dorsal most embryonic cells in a domain of presumptive notum surrounding the dorsal midline, and at the dorsal anterior margin of the eye disc (Heitzler et al., 1996; Ramain et al., 1993; Maurel-Zaffran and Treisman, 2000). Pnr is not expressed in the first instar larval eye-antennal imaginal disc (Fig. 1A). In the eye-antennal disc, pnr expression begins either in the late first instar stage or in the early second instar stage (Singh and Choi, 2003). In the early second instar eye-antennal imaginal disc, pnr expression begins in 5–7 cells in the dorsal margin of antenna and head region of the eye-antennal disc (Fig. 1A). During mid- to late-second instar of larval eye development, pnr begins to express in 30–35 cells (Fig. 1B). During the late second instar stage of larval eye development, pnr expression spreads to 80–100 cells in the dorsal eye domain (Fig. 1C). Interestingly, pnr expression is not seen in the disc proper cells, which differentiate to retinal photoreceptor cells in the eye (Fig. 1C’). Furthermore, pnr expression is restricted to the peripodial membrane on the dorsal eye margin (Pereira et al., 2006). Rarely a few disc proper cells (anterior to the morphogenetic furrow) at the border with peripodial membrane in the eye disc show pnr expression (Fig. 1C”). As the larva progresses into its third instar stage, pnr extends throughout the dorsal-most region of the head and antenna. The pnr expression in the third instar eye-antennal imaginal evolves into 4–5 rows of cells on the dorsal eye margin (Fig. 1D). Pnr expression overlaps with the MF but does not

![Fig. 1](image-url). Pnr expression is restricted to the peripodial membrane (PM) of the dorsal eye margin. (A) pnr expression (pnr Gal4 drive UAS-GFP, Singh and Choi, 2003; Singh et al., 2005a,b) is absent in the first instar eye-antennal imaginal disc whereas Wg (red) is expressed in the entire eye disc. Note that pnr expression in the brain at this stage is seen. (B) In the second instar eye-antennal imaginal disc, pnr expression (green) is initiated in 15–20 cells on the dorsal eye margin and Wg (red) is expressed laterally on both dorsal and ventral eye margins. At this stage, Hth (blue) expression is present in the entire eye disc. (C, C’, C”) In the early third instar eye-antennal disc, pnr expression in the dorsal eye margin is restricted only to the peripodial membrane (PM) whereas Hth (blue) is also expressed in peripodial membrane (PM) of the eye-antennal disc. (C) pnr (green) expression at this stage is absent in the disc proper (DP). Hth (blue) expression begins to retract with the initiation of MF and stays anterior to the furrow (Pai et al., 1998; Bessa et al., 2002; Singh et al., 2002). (C”) Pnr expression is restricted to the peripodial membrane (PM) specific cells on the dorsal eye margin. (D) In the late third instar eye-antennal imaginal disc, pnr (green) expression is restricted to the dorsal eye margin whereas Wg (red) expression is restricted to the dorsal and ventral eye margins. Hth (Blue) is expressed in rings in the proximal region of antenna and expressed both in the dorsal and ventral part of the disc proper anterior to the furrow. Dashed lines indicate the approximate midline, the border between D (Dorsal) and V (ventral) eye. All the eye-antennal imaginal discs and the adult eyes are organized as Dorsal (D) up and the ventral (V) down. Markers for immunostaining are shown in color labels. (AN: Antenna).
coincide with retinal cells as it is expressed only in the peripodial membrane.

Wg expression, which acts downstream to Pnr, is localized to the dorsal as well as the ventral eye margins in the disc proper cells (Fig. 1B–E). Wg is also expressed throughout all larval stages in the PM (Cho et al., 2000). Wg is expressed in both the dorsal and ventral compartments, but expression in the dorsal is constant throughout all larval stages in the peripodial membrane. Wg is controlled by pnr in the peripodial membrane only; the regulation of its disc proper expression in the dorsal eye disc is not fully understood as of yet. In the first instar and early second instar eye disc, hth is expressed in the entire eye disc (Fig. 1B; Singh et al., 2002; Bessa et al., 2002). The expression of hth in the disc proper (DP) begins to retract in late second instar (Fig. 1C) whereas hth is expressed in the entire peripodial membrane (PM). In late third instar stage, hth expression stays anterior to the MF in the late third instar stage (Fig. 1D; Singh et al., 2002; Bessa et al., 2002).

Loss-of-function clones of pnr show four different phenotypes

We employed the genetic mosaic approach to generate loss-of-function clones of pnr in the developing eye-antennal imaginal disc (Xu and Rubin, 1993). We used the pnr<sup>meta</sup> mutant, a null allele which has a deletion of all but 9 amino acids of the coding region (Ramaiah et al., 1993; Heitzler et al., 1996), to generate the genetic mosaic clones. Mutant clones were generated in the eye using the FLP/FRT system where Flippase is under the control of an eye-specific enhancer of eyeless (ey). (Quiring et al., 1994). Since pnr is expressed in the dorsal eye, the loss-of-function clones of pnr that are located only in the dorsal eye margin exhibit phenotypes. The loss-of-function clonal phenotypes of pnr can be classified into four different categories:

Non-autonomous dorsal eye enlargement

Loss-of-function clones of pnr in the dorsal eye result in an ectopic eye field or enlargement of the existing eye field comprising of differentiating photoreceptor neurons. These eye field enlargements or ectopic eye fields can even extend anterior to the furrow only on the dorsal eye margin (Fig. 2A; marked by white dotted line). Further, these eye enlargement phenotypes are non-autonomous, which include both mutant cells (lack GFP reporter, marked by dotted boundary in Fig. 2A) as well as the adjoining wild-type cells (GFP positive). In the adult eyes, the pnr clones were marked by absence of the mini-white reporter gene, which is involved in the pigment uptake in the eye (Sun et al., 1995). These clones resulted in either enlargement of the pre-existing dorsal eye field or generation of a de novo ectopic eye field in the dorsal head cuticle (Fig. 2B; marked by black dotted line). These ectopic eye fields did not arise exclusively within the pnr mutant clones, but also contained a domain of the wild-type cells (marked by dark red pigment). The phenotype of these clones resembled the loss-of-function clone phenotypes of pnr described earlier (Maurel-Zaffran and Treisman, 2000; Singh and Choi, 2003). These phenotypes were explained to be due to generation of a de novo equator between the pnr<sup>−</sup> and pnr<sup>+</sup> cells. The frequency of these clones is around ~8.3% of the total pnr loss-of-function clones (Table 1). Strikingly, we observe only the larger size clones in this category.

Autonomous dorsal eye enlargement

Loss-of-function clones of pnr in this category showed an ectopic field of differentiating photoreceptors anterior to the morphogenetic furrow in the dorsal eye domain. Unlike the clones of previous category, the ectopic eye field in these clones was autonomous (restricted within the pnr loss-of-function clones) (Fig. 2C, clonal boundary marked by white dotted line). In the adult flies, these clones resulted in the formation of an ectopic eye field in the dorsal head cuticle anterior to the eye field (Fig. 2D, black dotted line). The ectopic eye field in the clones of this category was devoid of any wild-type pnr<sup>+</sup> cells, clearly suggesting that pnr loss-of-function led to the generation of ectopic eyes. The frequency of these clones was nearly 12.6%, which comprises of both the smaller (7.0%) as well as the bigger (5.6%) clones (Table 1). Some of these clones were accompanied by cuticle enlargement in the head.

Absence of dorsal eye enlargement

Unlike the previous two categories of pnr clones (Fig. 2A–D), loss-of-function clones in this category does not result in any ectopic

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**Fig. 2.** Loss-of-function of pnr exhibits a range of eye enlargements and antennal duplications in the dorsal eye. (A, B) Loss-of-function clones of pnr in the dorsal eye margin (marked by the absence of GFP (green) in the eye-antennal imaginal disc and absence of the mini-white reporter (red) in the adult eye) results in a non-autonomous ectopic eye enlargement as seen in the eye-antennal imaginal disc and in the adult eye. The ectopic eye enlargements are not restricted within the clone. However, they extend both in the wild-type as well as in the pnr mutant cells of the eye-antennal disc. Note that the dorsal clone boundary is marked by white dotted line in the eye disc and by black dotted line in the adult eye. (C, D) Loss-of-function of pnr in the dorsal eye results in an autonomous ectopic dorsal eye anterior to the normal eye field. These ectopic eyes are restricted to within the clones. Note that not all the cells of the pnr loss-of-function clone differentiate to the photoreceptors. (E, F) Loss-of-function clones of pnr in the dorsal eye have no effect on the eye field as seen in the eye disc and the adult eye. All these clones were restricted to the disc proper. (G, H) Loss-of-function clones of pnr in the antenna results in duplication of the antennal field as seen in (G) the eye-antennal disc and (H) the adult head. (H) Scanning electron microscopy (SEM) of the adult head showing antennal duplication and dorsal eye enlargement (Magnification ×180). Note that only a few pnr loss-of-function clones show both dorsal eye enlargements along with the antennal duplication.
dorsal eye enlargement (Fig. 2E). Interestingly, even though these clones span both the anterior as well as the posterior regions of the morphogenetic furrow in the dorsal eye-antennal imaginal disc but did not result in any ectopic eyes in the eye disc (Fig. 2E) as well as in adult flies (Fig. 2F). The frequency of these clones is nearly 75.3% (Table 1). We found that these clones unlike the clones from the previous two categories are restricted only to the disc proper in the dorsal eye; a domain where pnr is normally not expressed (Fig. 1).

Table 1

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Large clones with dorsal eye enlargements</th>
<th>Small clones with dorsal eye enlargements</th>
<th>No dorsal eye enlargements</th>
<th>Antennal duplication</th>
<th>Total flies (with clones)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clones with Non-autonomous dorsal eye enlargement, Class I</td>
<td>34 (8.3%)</td>
<td></td>
<td></td>
<td></td>
<td>34</td>
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<tr>
<td>Clones with autonomous dorsal eye enlargement, Class II</td>
<td>23 (5.6%)</td>
<td>29 (7.0%)</td>
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<td>52</td>
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<tr>
<td>No dorsal eye enlargement, Class III</td>
<td></td>
<td></td>
<td>308 (75.3%)</td>
<td>15 (3.6%)</td>
<td>323</td>
</tr>
<tr>
<td>Antennal duplication, Class IV</td>
<td>(10)*</td>
<td>(4)*</td>
<td></td>
<td></td>
<td>409</td>
</tr>
<tr>
<td>Grand total</td>
<td>(10)*</td>
<td>(4)*</td>
<td>308 (75.3%)</td>
<td>15 (3.6%)</td>
<td>409</td>
</tr>
</tbody>
</table>

* The flies in this category showed both antennal duplications dorsal eye enlargements of large and small size.

Antennal duplication

Loss-of-function clones of pnr in the antenna region of the eye-antennal imaginal disc, results in the duplication of antennal field (Fig. 2G). Interestingly, most of the antennal duplications were accompanied with ectopic eye enlargements (Fig. 2H). However, in some of these clones only antennal duplication were observed. These clones led to duplication of the ventral head structures such as antenna and maxillary palps in the dorsal head (Fig. 2H; Maurel-Zaffran and Treisman, 2000; Pichaud and Casares, 2000; Singh et al., 2005b). The frequency of these clones was ~3.6% (Table 1). Thus, our analysis of loss-of-function clones suggests that pnr may be involved in the suppression of eye fate.

Pnr suppresses the eye fate in the dorsal eye

To test the role of pnr in eye fate determination, we used the Gal4/UAS system to misexpress pnr in the eye (Brand and Perrimon, 1993). We used a UAS-pnr^Zf4 construct that behaves like wild-type pnr in the absence of U-shaped (ush) function (Haenlin et al., 1997; Maurel-Zaffran and Treisman, 2000; Fossett et al., 2001; Singh and Choi, 2003). Ush encodes a zinc finger protein that dimerizes with Pnr and acts as a negative regulator of pnr transcriptional activity (Haenlin et al., 1997). Since ush is not expressed in the eye-antennal imaginal disc, the UAS-pnr^Zf4 construct behaves in a wild-type fashion in the eye (Maurel-Zaffran and Treisman, 2000; Fossett et al., 2001). We used an ey-Gal4 driver that drives the expression of UAS-GFP transgene in the entire eye disc, which comprises of differentiating retinal neurons (posterior to MF, marked by white arrowhead) as well as the region forming the prospective head cuticle (anterior to MF) (Fig. 3A; Singh et al., 2005a). Misexpression of pnr in the entire eye disc using ey-Gal4 (ey>pnrZf4) results in the complete loss of eye field as evident from the absence of neuronal marker Elav whereas the size of antennal field is not affected (Fig. 3C). The misexpression of pnr in the entire eye (ey>pnrZf4) results in the adult flies with highly reduced eye field or what we refer to as the “no-eye” phenotypes (Fig. 3D) as compared to the wild-type eyes (Fig. 3B). To test if there is any domain specific response of pnr misexpression, we employed bi-Gal4 driver, which drives the expression of UAS-GFP transgene (bi>GFP) on both dorsal and ventral margins of the developing eye-antennal imaginal disc (Fig 3E; Calleja et al., 1996; Singh et al., 2002, 2004). Misexpression of pnr using bi-Gal4 (bi>pnrZf4) suppressed the eye fate on both dorsal and ventral eye margins as evident from the absence of Elav expression (Fig. 3F; white arrows). This suggests that pnr upon misexpression suppresses the eye fate, irrespective of dorsal or ventral domains. Random gain-of-function clones of pnr in the eye using UAS-pnr^Zf4 (marked by GFP reporter) caused suppression of photoreceptors as evident from the absence of Elav positive cells in the eye disc (Fig. 3G, G’) as well as in the adult eye (Fig. 3H). In addition to the small eye phenotypes seen in the gain-of-function clone of pnr, we observed necrosis as evident from presence of dark spots in the adult eye (Fig. 3H). These results suggest that pnr can suppress the eye fate. The frequency of pnr gain-of-function clones was extremely low in the eye disc as well as the adults probably due to issues with cell survival.

We then disrupted Pnr function using dominant negative pnr (UAS-pnr^ENS) (Fu et al., 1998; Klinedinst and Bodmer, 2003) where the construct contains the repressor domain from the Engrailed transcription factor (EnR, amino acid 2-298) (Jaynes and O’Farrell, 1991) and the two N-terminal zinc-finger domains from pnr (amino acids 153-293) (Ramain et al., 1993). Disrupting pnr function in the entire eye-antennal imaginal disc all along during eye development by misexpression of pnr^ENS (ey>pnr^ENS) resulted in a small group of Elav positive retinal cells in the eye field (Fig. 3C), and a highly reduced eye in the adult fly (Fig. 3H). However, there was no affect on the developing antennal field (Fig. 3G, H). This suggests that during early eye development when DV patterning is being established pnr function is crucial for eye development. However, misexpression of pnr^ENS on both dorsal and ventral eye margins using bi-Gal4 (bi>pnr^ENS) caused enlargement of only the dorsal eye (Fig. 3H, arrow). In bi>pnr^ENS background, pnr function was abolished only in a few subset of cells within the endogenous pnr expression domain in the peripodial membrane of the dorsal eye. These dorsal eye enlargement phenotypes further substantiated the possibility that pnr may suppress retinal determination in the dorsal eye margin.

Pnr downregulates the retinal determination genes to suppress the eye

To address the role of pnr in retinal determination, we checked the expression of members of the retinal determination (RD) gene pathway in the loss-of-function clones of pnr in eye-antennal imaginal disc. The loss-of-function clones of pnr (marked by absence of the GFP reporter) that exhibit enlargement of the dorsal eye showed no ectopic induction of Ey (Fig. 4A, A’, A”). It has been shown that Ey is expressed in undifferentiated retinal precursor cells early in eye development and after the onset of photoreceptor differentiation; Ey continues to express in the undifferentiated retinal precursor cells anterior to the MF (Quiring et al., 1994; Lee and Treisman, 2001a,b; Singh et al., 2002; Bessa et al., 2002). Although Ey expression was not affected, the loss-of-function clones of pnr which caused ectopic dorsal eye enlargements showed ectopic induction of Eya (Fig. 4B, B’, B”), which acts downstream to Ey. The loss-of-function clones of pnr in the ventral domain of the eye did not exhibit any affect on wild-type Eya expression. During the late first instar stage, eyu begins expression in the eye region of the disc. A short time after eyu expression, so, dac, and ey are expressed in the late second instar stage in the region posterior to the MF (Bonini et al., 1993; Cheyette et al., 1994; Jang et al., 2003; Kenyon et al., 2003; Mardon et al., 1994). After the MF begins expression of eyu and so are restricted to the area within and posterior to
the MF (Bonini et al., 1993; Cheyette et al., 1994). The expression of dac is restricted to the MF in the area that directly precedes the MF and continues in R1, R6 and R7 for a few columns posterior to the MF and sharply disappears after that domain (Mardon et al., 1994; Tavsanli et al., 2004). The loss-of-function clones of pnr in the dorsal eye showed ectopic induction of So (Fig. 4C, C', C"; white arrows) and Dac (Fig. 4D, D', D"; white arrows). Dac is expressed downstream to eya, and so (Chen et al., 1997). The ventral eye clones did not exhibit any effect on the expression of RD genes. These results suggest that eya, a gene expressed in undifferentiated cells, is not induced in ectopic dorsal eye enlargement whereas the downstream RD genes like eya, so, dac that are expressed in differentiating photoreceptor neurons are upregulated in the pnr loss-of-function clones. The pnr loss-of-function clones which only result in antennal duplications does not affect RD genes expression (data not shown). These results suggest that pnr may act downstream of ey to suppress retinal determination on the dorsal eye margin. Furthermore, since pnr is expressed in the peripodial membrane on the dorsal eye margin, these loss-of-function clonal phenotypes of pnr suggest that pnr blocks retinal determination in the peripodial membrane of the dorsal eye margin. Thus, pnr may promote head specification.

We tested this hypothesis by checking RD gene expression in bi>pnrd4 background where pnr misexpression suppresses the eye both on dorsal and ventral eye margins (Fig. 3F). Interestingly, Ey was present on both dorsal and ventral margins (Fig. 4E, E'; white arrowheads). However, the expression of other RD genes like Eya (Fig. 4F, F'; white arrowheads), So (Fig. 4G, G'; white arrowheads), and Dac (Fig. 4L, L'; white arrowheads) was downregulated on both the dorsal as well as the ventral eye margins of highly reduced eye-antennal imaginal disc. Dac expression anterior to the MF is not affected (Fig. 4I, I'). These results strongly suggest that pnr suppresses retinal determination by blocking Eya, So, and Dac expression.

Pnr suppresses eye by induction of its downstream target Wg

In order to test if, the misexpressed pnr is functional in the eye disc; we tested the levels of Wg as a functional read out of pnr in the eye. Pnr acts upstream to the signaling molecule Wg, that suppresses the eye fate (Ma and Moses, 1995; Treisman and Rubin, 1995; Lee and Treisman, 2001a,b). Wg is expressed on the antero-lateral margin of both dorsal and ventral eye (Fig. 1D). Misexpression of pnr in the eye disc (ey>pnrd4) results in the suppression of eye (Fig. 3B). We found that misexpression of pnr on both the dorsal and the ventral margin (bi>pnrd4) of eye disc results in robust induction of Wg along with a strong suppression of the eye on both the dorsal as well as the ventral margins (Fig. 5A, A'). bi>pnrd4 showed similar eye suppression
phenotypes on both the dorsal and ventral eye margins in the adult eye (Fig. 5B). This phenotype is similar to the misexpression of Wg on both dorsal and ventral margins (bi\textgreater pnrD4) that results in suppression of the eye on both dorsal as well as ventral margins (Singh et al., 2002). Targeted misexpression of pnr using (ey\textgreater pnrD4) results in a "no-eye" phenotype by induction of Wg in the entire eye (Fig. 5C). These phenotypes are comparable to the ectopic induction of Wg in the eye (ey\textgreater wgl) (data not shown, Singh et al., 2002). Interestingly, the ectopic eye induction and Wg downregulation did not cover the entire loss-of-function clone of pnr in the dorsal eye (Fig. 5D, marked by the white dotted line). But the Wg expression was present within the clone juxtaposed to the wild-type wg expression domain in the head region (Fig. 5D). This Wg expression phenotype can be explained as rescue of Wg within the mutant clone from the wild-type cells due to the secretary nature of Wg. The loss-of-function clones of pnr in the disc proper (DP) did not result in ectopic eye enlargements and showed no affect on Wg expression (Fig. 5F, F'). In some of the larger loss-of-function clones of pnr, which extend from the dorsal eye margin into the
Pnr eye suppression function in the dorsal eye margin is independent of hth

In order to understand the mechanism by which pnr suppresses the eye fate, we tested whether loss of pnr function induces hth. Misexpression of hth on both dorsal and ventral eye margins (bi–hth) results in suppression of the eye on both dorsal and ventral eye margins (Fig. 6A, A', arrows) as also seen in bi–pnrD4 eye–antennal discs (Fig. 3F). Therefore, we tested levels of Hth in bi–pnrD4 eye–antennal disc. Interestingly, Hth was induced both on the dorsal as well as the ventral eye margins (Fig. 6B, B', arrows). This raises the possibility that pnr may suppress the eye development by inducing downstream hth. Therefore, if hth is downstream to pnr in the dorsal eye, then loss-of-function of hth must be similar to pnr loss-of-function phenotypes. We generated loss-of-function clones of hth in the eye disc. We found that the loss-of-function clones of hth induced at any time during larval development autonomously induced ectopic eyes only in the ventral head capsule (Fig. 6C, C'; marked by white dotted line, Pai et al., 1998; Pichaud and Casares, 2000). However, hth loss-of-function clones did not show any ectopic dorsal eye enlargements as seen in the pnr loss-of-function clones (Fig. 2). Thus, unlike gain-of-function of hth that corresponds to the gain-of-function of pnr (Fig. 3), the loss-of-function of hth does not match pnr loss-of-function clonal phenotypes. This result rules out the possibility of pnr acting upstream of hth in the dorsal eye. We studied the expression of Hth in the pnr loss-of-function clones and found that Hth expression was not affected in loss-of-function clones of pnr showing ectopic eye enlargements (Fig. 6D, D'). Hth marks the undifferentiated retinal precursor cells anterior to the furrow (Fig. 1; Pai et al., 1998; Bessa et al., 2002). In pnr loss-of-function clones, where no ectopic eye enlargements were seen, Hth expression was not affected (Fig. 6E, E'). Interestingly, Hth expression was induced in pnr loss-of-function clones where the duplication of antennal region took place (Fig. 6F, F'). The duplication of antennal region represents the ventral structures in head capsule (Casares and Mann, 1998). Since hth is expressed in the proximal domains of antennal field there is ectopic induction of hth when duplication of the antennal field and cuticle enlargement occurs. Thus, our results suggest that pnr does not directly affect the hth expression in the dorsal eye.

We employed a candidate gene approach and looked for the genes which might affect the dorsal eye patterning. Homeotic gene teashirt (tsh) shows an asymmetric response on the dorsal and ventral eye margins. In the early eye, tsh is expressed in the entire disc (Singh et al., 2002; Bessa et al., 2002). In the second instar eye–antennal imaginal disc, Tsh expression begins to retract anteriorly (Fig. 7A), and in the third instar eye-disc Tsh is expressed in the retinal precursor cells anterior to the MF (Fig. 7A, B; Bessa et al., 2002; Singh et al., 2002). We tested expression of tsh in the loss-of-function clones of pnr in the dorsal eye. We found that pnr loss-of-function clones, which exhibit dorsal eye enlargement also exhibit ectopic induction of Tsh (Fig. 7C, C') as well as the tsh reporter y, w; tshA8 (Fig. 7C, C'). It suggests that pnr might suppress tsh expression at the transcription level. In order to test whether pnr suppresses the eye by downregulating tsh, we generated pnr loss-of-function clones where tsh levels were reduced to 50% using a heterozygous combination of tsh, a null allele of tsh (Paisano et al., 1991). Interestingly, in these pnr loss-of-function clones where tsh function was reduced to half, we did not see any ectopic eye enlargement (Fig. 7D). Interestingly, although the dorsal clones did induce overgrowths/enlargement, these clones did not show any ectopic Elav expression. However, these clones exhibited strong Ey expression (Fig. 7D', D''). The adult eye phenotype of these clones is similar to the eye disc phenotype of lack of any dorsal eye enlargements (Fig. 7E). To further test our hypothesis that pnr affects tsh expression at the transcription level, we misexpressed pnr on the dorsal and ventral eye margins (bi–pnrD4) and checked the expression of the tsh reporter. We found that tsh expression was downregulated on both the dorsal and ventral eye margins (Fig. 7F, arrows). Since endogenous expression of pnr is restricted to the dorsal...
Fig. 6. pnr suppresses the eye fate at dorsal eye margin independent of hth. hth, a Meis class of gene (Rieckhof et al., 1997), acts as a negative regulator of the eye (Pai et al., 1998). Hth expression is restricted anterior to furrow in 10–15 cell wide domain and in entire peripodial membrane (Fig. 1). (A, A’) Misexpression of hth on both dorsal and ventral eye margin (bi–hth) results in the suppression of eye fate on both dorsal and ventral margin of the eye disc as evident from (A’) suppression of Elav (marked by white arrows). (B, B’) Misexpression of pnr on both dorsal and ventral eye margin (bi–pnrD4) results in suppression of eye fate on both dorsal and ventral eye margin (marked by white arrows), which is accompanied by induction of Wg (green) as well as Hth (red; white arrows). (C, C’) Loss-of-function clone of hth in the ventral eye results in the eye enlargement as evident from Elav expression (marked by white dotted line). Note that the dorsal eye clones do not exhibit any phenotype. (D–F) In loss-of-function clones of pnr, (D, D’) which result in dorsal eye enlargement (marked by white dotted line) or (E, E’) which do not exhibit dorsal eye enlargement (marked by white dotted line), Hth (red) expression stays anterior to the furrow as seen in the wild-type eye disc. Loss-of-function clones of pnr in the antennal disc which results in the duplication of antennal field exhibit ectopic Hth expression in the duplicated antennal disc (marked by white dotted line). Note that hth is expressed in the proximal region of the antennal disc.

We have addressed a basic question pertaining to regulation of patterning, growth and differentiation of the developing eye field. Our results provide an important insight into the role of pnr, a gene known to confer dorsal eye identity during axial patterning of the eye (Maurel-Zaffran and Treisman, 2000; Pichaud and Casares, 2000; Singh and Choi, 2003). We and others have shown that the onset of pnr expression during early eye development results in the generation of dorsal lineage in the eye. It results in the formation of a DV boundary (equator), which triggers N signaling at the border of the dorsal and ventral compartments to initiate growth and differentiation (Cho and Choi, 1998; Dominguez and de Celis, 1998; Papayannopoulos et al., 1998; Singh et al., 2005b).

Earlier, we have tested the spatial as well as temporal requirement for the genes controlling ventral eye growth and development (Singh and Choi, 2003). During early eye development, prior to the onset of pnr expression in the dorsal eye, entire early eye primordium is ventral in fate (Singh and Choi, 2003). Removal of function of genes controlling ventral eye development prior to the onset of pnr expression, result in complete elimination of the eye field whereas later when pnr starts expressing, the eye suppression phenotype gets

Two phenotypes are complementary to the pnr loss-of-function phenotype. Misexpression of pnrD4 in the dorsal eye results in the suppression of eye fate on both dorsal and ventral margin of the eye disc as evident from (A’) suppression of Elav (marked by white arrows). (B, B’) Misexpression of pnr on both dorsal and ventral eye margin (bi–pnrD4) results in suppression of eye fate on both dorsal and ventral eye margin (marked by white arrows), which is accompanied by induction of Wg (green) as well as Hth (red; white arrows). (C, C’) Loss-of-function clone of hth in the ventral eye results in the eye enlargement as evident from Elav expression (marked by white dotted line). Note that the dorsal eye clones do not exhibit any phenotype. (D–F) In loss-of-function clones of pnr, (D, D’) which result in dorsal eye enlargement (marked by white dotted line) or (E, E’) which do not exhibit dorsal eye enlargement (marked by white dotted line), Hth (red) expression stays anterior to the furrow as seen in the wild-type eye disc. Loss-of-function clones of pnr in the antennal disc which results in the duplication of antennal field exhibit ectopic Hth expression in the duplicated antennal disc (marked by white dotted line). Note that hth is expressed in the proximal region of the antennal disc.

eye margin, our results suggest that pnr suppresses the tsh expression in the dorsal eye. We further tested pnr and tsh interaction, by misexpressing pnr on both dorsal and ventral eye margins in a tsh+/+ heterozygous background (bi–pnrD4; tsh+/+). In this tsh heterozygous background, misexpression of pnr strongly enhances the eye suppression phenotype on both the dorsal and the ventral eye margins (Fig. 7G) as compared to bi–pnrD4 alone (Fig. 7F). These results suggest that pnr may suppress tsh at the dorsal eye margin. Misexpression of tsh on the dorsal and the ventral eye margin (bi–tsh) results in dorsal eye enlargement and ventral eye suppression (Fig. 7H; Singh et al., 2002). This phenotype is complementary to the pnr loss-of-function phenotype. Misexpression of tsh RNAi using bi-Gal4 results in phenotype that is complementary to bi–tsh phenotype in the dorsal eye (data not shown). We therefore tested whether misexpression of tsh can rescue the pnr misexpression phenotype. Misexpression of both pnr and tsh (bi–tsh + pnrD4) resulted in the lethality as early as the first instar larval stage. We therefore, misexpressed ara, a downstream target of pnr and a member of Iro-C complex, with tsh on the dorsal and ventral eye margins (bi–tsh + ara), which resulted in the enlargement of the eye on the dorsal eye margin (Fig. 7C; Singh et al., 2004). We further tested the hypothesis that pnr downregulates tsh in the dorsal eye to suppress eye fate. Misexpression of tsh in the dorsal eye using a pnr-Gal4 driver (pnr–tsh) resulted in the enlargement of eye on the dorsal margin (Fig. 7J). Lastly, we tested whether reducing pnr levels affects the bi–tsh phenotype. In heterozygous pnr background we misexpressed tsh (bi–tsh; pnr+/+) and found that it results in the dorsal eye enlargements (Fig. 7K). These eye enlargements were similar to that of the bi–tsh alone (Fig. 7H). This suggests that tsh acts downstream to pnr and therefore levels of tsh are crucial for the dorsal eye enlargement phenotype. Thus, pnr suppresses the eye development on the dorsal eye margin by suppressing tsh.

Discussion

We have addressed a basic question pertaining to regulation of patterning, growth and differentiation of the developing eye field. Our results provide an important insight into the role of pnr, a gene known to confer dorsal eye identity during axial patterning of the eye (Maurel-Zaffran and Treisman, 2000; Pichaud and Casares, 2000; Singh and Choi, 2003). We and others have shown that the onset of pnr expression during early eye development results in the generation of dorsal lineage in the eye. It results in the formation of a DV boundary (equator), which triggers N signaling at the border of the dorsal and ventral compartments to initiate growth and differentiation (Cho and Choi, 1998; Dominguez and de Celis, 1998; Papayannopoulos et al., 1998; Singh et al., 2005b).

Earlier, we have tested the spatial as well as temporal requirement for the genes controlling ventral eye growth and development (Singh and Choi, 2003). During early eye development, prior to the onset of pnr expression in the dorsal eye, entire early eye primordium is ventral in fate (Singh and Choi, 2003). Removal of function of genes controlling ventral eye development prior to the onset of pnr expression, result in complete elimination of the eye field whereas later when pnr starts expressing, the eye suppression phenotype gets
restricted only to the ventral eye (Singh and Choi, 2003; Singh et al., 2005a). These studies suggested that pnr plays an important role in dorso-ventral (axial) patterning. However, the role of dorsal selector pnr in retinal determination was unknown.

**Pnr suppresses the eye fate**

Loss-of-function clones of pnr in the dorsal eye exhibit eye enlargement (Fig. 2A; Maurel-Zaffran and Treisman, 2000; Pichaud and Casares, 2000; Singh and Choi, 2003). It was suggested that when pnr function was abolished in the dorsal eye using loss-of-function clones, it results in the change of dorsal eye fate to ventral. This results in generation of a de novo equator, the border between dorsal and ventral half of the eye, which triggers ectopic N signaling to promote growth and cell proliferation. The same premise was used to explain the gain-of-function phenotype of pnr in the eye. Misexpression of pnr in the entire eye (ey-pnr) generates a completely dorsalized eye field due to lack of N downregulation (Fig. 3C, D; Maurel-Zaffran and Treisman, 2000). Here, we addressed another possibility to see if pnr suppresses the eye fate upon misexpression in the entire eye as evident from the “no-eye” phenotype.

To test if pnr suppresses the eye fate, we misexpressed pnr both in the dorsal and ventral (DV) eye margins of the eye using a bi-Gal4 driver (bi-pnr) (Fig. 3F). The rationale was if pnr misexpression (bi-pnr) will assign a dorsal fate on the margin of ventral eye. Thus, by this logic, it would result in regeneration of a de novo equator on the ventral eye margin, which should manifest as eye enlargements in the ventral eye. The argument was based on the premise that was employed to explain that loss-of-function clones of pnr in the dorsal eye generated a new equator and led to the dorsal eye enlargement. We did not observe any ventral eye enlargements in bi-pnr) eye disc (Fig. 3F). Instead we saw suppression of the eye on both the dorsal and ventral eye margin, suggesting that pnr acts upstream of pnr.

**Fig. 7.** Pnr suppresses the eye fate by downregulating teashirt (tsh) in the dorsal eye margin. Tsh, a Hox gene (Fasano et al., 1991), exhibits Dorso-ventral (DV) asymmetric function in the eye (Singh et al., 2002). pnr expression initiates in early second instar eye-antennae disc (Singh and Choi, 2003). (A) In the late second instar, pnr expression evolves and is restricted to 50-100 cells of the dorsal eye margin. At this stage when MF has just initiated, Tsh is expressed anterior to the furrow (MF). (B) In the third instar eye imaginal disc, pnr is expressed on the dorsal eye margin whereas tsh is expressed in the eye disc anterior to the furrow. (C-C”) Loss-of-function clone of pnr in the dorsal eye marked by the loss of GFP reporter (marked by white dotted line) exhibit (C’) ectopic localization of Tsh protein, and (C”) ectopic expression of tsh reporter (tsh-Gal4) in the dorsal eye. (D-D”) Loss-of-function clones of pnr (marked by loss of GFP), where tsh function is reduced to half using a heterozygous background of tsh null allele (tsh+/+), (D”) exhibit outgrowth on the dorsal eye margin which is positive for Ey expression but there is no ectopic eye enlargement as evident from Elav expression. Note that eye size is reduced on both margins. (E) Misexpression of ey in the ventral eye margin which is positive for Ey expression but there is no ectopic eye enlargement as evident from (D”) absence of neuronal marker Elav expression. The dorsal overgrowth exhibits robust expression of Ey, a marker for undifferentiated retinal precursor cells. (F) Loss-of-function clone of pnr in the tsh heterozygous background marked by the loss of mini-white reporter (red: clonal boundary marked by black dotted line) results in the absence of eye enlargement in the adult eye. These results suggest that pnr eye suppression function is mediated through downregulation of tsh. (F) Misexpression of pnr on the dorsal and the ventral eye margin, bi-pnr(1-4), results in the suppression of tsh reporter on both dorsal and ventral eye margin (white arrowhead) along with the suppression of eye as evident from Elav (blue) expression. Note that eye size is reduced on both margins. (G) Misexpression of pnr in the dorsal and ventral eye margin in tsh heterozygous background (tsh+/+; bi-pnr 1-4) exhibits strong suppression of eye expression resulting in a highly reduced eye. Note that bi-pnr(1-4) alone (F) shows suppression of eye both on the dorsal and the ventral eye margin. However, the size of bi-pnr(1-4) eye imaginal disc size is extremely reduced as compared to bi-pnr(1-4) alone. (H) Misexpression of tsh on DV margin (bi-tsh) results in the suppression of eye on the ventral margin whereas eye enlargement in the dorsal eye (Singh et al., 2002). Misexpression of both tsh and pnr on DV margin results in early lethality. Therefore, we misexpressed pnr downstream target ara with tsh. (I) Misexpression of tsh with dorsal eye selector ara, a downstream target of pnr, on DV margin using bi-Gal4 (bi-tsh + ara) results in the enlargement on both dorsal and ventral eye margins. Misexpression of tsh and ara on DV margin results in strong dorsal eye enlargements. (J) Misexpression of tsh using pnr-Gal4 driver (pnr-tsh) results in the enlargement of the dorsal eye. (K) Misexpression of tsh in the heterozygous pnr background results in the dorsal eye enlargement. However, these eye enlargements are not bigger than what is seen in (I) bi-tsh or (J) pnr-tsh, suggesting that pnr acts upstream of tsh.
Pnr suppresses the Retinal Determination (RD) genes function

Since pnr suppresses the eye fate, it is possible that it may be involved in regulation of expression of genes of the core retinal determination machinery. Loss-of-function of pnr in the dorsal eye clones results in the eye enlargements as evident from Elav positive cells but it does not induce ectopic Ey (Fig. 4A). Ey expression evolves during eye development and is localized anterior to the morphogenetic furrow in retinal precursor cells and is downregulated and degraded posterior to the furrow in differentiation retinal neurons (Quiring et al., 1994; Halder et al., 1995, 1998; Baonza and Freeman, 2002; Kango-Singh et al., 2003; Lee and Treisman, 2001a,b). We found that in the loss-of-function clones of pnr in the eye, the expression of retinal determination pathway members like Eya, So and Dac, which act downstream to Ey, was ectopically induced (Fig. 4B–D). In the converse situation, where pnr was misexpressed on both dorsal and ventral eye margins (bi–pnrD4), we observed ectopic induction of Ey on both the dorsal and the ventral margins (Fig. 4E) whereas the expression of Eya (Fig. 4F), So (Fig. 4G) and Dac (Fig. 4H) were suppressed. Thus, misexpression of pnr in the eye prevents the photoreceptor differentiation irrespective of the dorsal or ventral domain. Based on these results we can propose that pnr suppresses the eye fate on the dorsal eye margin by downregulating RD genes like eya, so and dac (Fig. 8).

Since ey is responsible for the specification of the eye field and marks the retinal precursor cells, it suggests that pnr does not affect the eye field formation or specification. In fact pnr affects expression of RD genes eya, so and dac (Fig. 4), which acts downstream to ey, and are involved in retinal determination (Kango-Singh et al., 2003; Pappu and Mardon, 2004; Silver and Rebay, 2005; Kumar, 2009). Our results suggest that pnr suppresses retinal determination genes. Since endogenous expression of pnr is restricted to the peripodial membrane of the dorsal eye margin (Fig. 1), it suggests that pnr may be involved in suppression of retinal determination on the dorsal peripodial membrane. Thus, our results suggest that pnr generally acts at the stage when photoreceptor differentiation is initiated with the formation of the morphogenetic furrow (MF) and promotes the dorsal head cuticle fate by suppressing retinal differentiation (Fig. 8).

Dual function of pnr during eye development

Based on our new findings and other previously published results, we propose that pnr may be required for two different functions during eye development: (1) axis determination during DV patterning and (2) suppression of the retinal determination process to define the dorsal eye field margin. These functions of pnr appear to be temporally controlled as DV axis determination takes place in late first- or early second- instar of eye development (Singh and Choi, 2003; Singh et al., 2005b) while suppression of the eye fate is evident in late second instar of larval development.

The axis determination function of pnr is required in the earlier time window. This is further validated by the loss-of-function clones of pnr of first category, which are bigger and exhibits non-autonomous dorsal eye enlargement phenotypes (Fig. 2A, B). These dorsal eye enlargements that are spanning both wild-type and pnr mutant cells in the eye disc conforms to the notion that when pnr is lost in a group of cells during early development, it fails to confer dorsal identity over the default ventral state. As a consequence de novo equator is generated which results in the ectopic dorsal eye enlargements (Maurel-Zaffran and Treisman, 2000; Pichaud and Casares, 2000; Singh et al., 2005b). Since these clones are always bigger (Table 1), suggesting that they might be formed earlier. The late function of pnr in suppression of retinal determination is validated both by the gain-of-function studies (Fig. 3) as well as the loss-of-function clones of second category, which are both bigger as well as smaller in size and are autonomous in nature (Fig. 2C, D; Table 1). These clones have ectopic dorsal eyes, which are restricted within the clones, thereby suggesting that absence of pnr function promotes ectopic eye formation in the dorsal eye margin. Thus, during the early second instar of development, before the onset of retinal differentiation, pnr is required for defining the dorsal lineage by inducing Wg and members of the Iro-C complex (Maurel-Zaffran and Treisman, 2000; Singh et al., 2005b). However, later during the late second-instar stage of eye development, when the morphogenetic furrow (MF) is initiated, pnr suppresses the photoreceptor differentiation at the dorsal eye margin. The endogenous expression of pnr in only the peripodial membrane of the dorsal eye margin further confirms this notion (Fig. 1; Pereira et al., 2006). Lack of phenotypes in pnr clones which are restricted to the disc proper (DP) alone verifies pnr localization (Fig. 2E, F). Thus, pnr defines the boundary between the eye field and the head cuticle on the dorsal margin. An interesting question will be to identify which gene is responsible for defining the ventral eye margin. In the ventral eye where pnr is not expressed, hh is known to suppress the eye fate (Pai et al., 1998; Pichaud and Casares, 2000). There is a strong possibility that hh may be involved in defining the boundary of eye field on the ventral eye margin between the disc proper and peripodial membrane.

Pnr induces Wg to suppress the eye development independent of hh

Since pnr induces Wg, it is expected that pnr may suppress the eye by induction of Wg (Fig. 8). Even though Wg signaling is responsible for suppression of photoreceptor differentiation on both dorsal and ventral eye margins (Ma and Moses, 1995; Treisman and Rubin, 1995;
Lee and Treisman, 2001a,b; Baonza and Freeman, 2002), its regulation is different on both dorsal and ventral eye margins (Pichaud and Casares, 2000; Maurel-Zaffran and Treisman, 2000). In the ventral eye, wg is involved in a feedback loop with hth to suppress the eye fate (Pichaud and Casares, 2000; Singh et al., 2002). In the dorsal eye, Pnr induces Wg signaling, which in turn induces the members of Iro-C complex, and ultimately these signaling interactions define the dorsal eye fate. Interestingly, it seems Pnr is not the sole regulator of Wg in the dorsal eye (Fig. 5). Since loss-of-function of hth does not exhibit phenotypes similar to the loss-of-function of pnr (Fig. 6) or wg (Treisman and Rubin, 1995; Ma and Moses, 1995), it is expected that the positive feedback loop regulation of Wg and hth as seen in the ventral eye margin does not hold true on the dorsal eye margin. We found that hth is not affected in the pnr clones that exhibit dorsal eye enlargements. Furthermore, when we made clones of hth in pnr heterozygous condition, we did not see any dorsal eye enlargements suggesting that hth and pnr do not interact. Thus, like others, our results also verified that hth is not involved in pnr mediated eye suppression on the dorsal eye margin (Fig. 8; Pichaud and Casares, 2000).

Eye suppression function of pnr is mediated through the suppression of tsh

It is known that the gain of function of tsh in the dorsal eye results in ectopic eye enlargement whereas gain of function of tsh in the ventral eye result in suppression of ventral eye (Singh et al., 2002, 2004). The dorsal eye enlargements seen in tsh gain-of-function is a phenotype similar to pnr loss-of-function in the dorsal eye. In pnr loss-of-function clones, we found that tsh was ectopically induced (Fig. 7C). Furthermore, when pnr loss-of-function clones were generated in a heterozygous background of the tsh null allele tsh/– CyO (Fasano et al., 1991), the dorsal eye enlargement phenotype was dramatically suppressed and no longer observed. Interestingly, we found that dorsal enlargements were there but were not accompanied with ectopic eyes as evident from absence of Elav expression (Fig. 7D). All these dorsal enlargements were showing strong Ey expression. Among 500 flies counted we found only two flies that showed subtle dorsal eye enlargements. Interestingly, we also found that in pnr loss-of-function clones the mini-white reporter gene under tsh was ectopically induced (data not shown). The loss-of-function phenotypes of pnr were more pronounced when tsh was misexpressed in the clones. Thus, pnr expressed in the dorsal peripodial membrane may suppress tsh in the dorsal eye to suppress the eye fate (Fig. 8). Interestingly, tsh is known to act upstream of ey, so and dac (Pan and Rubin, 1998). Thus, the dorsal eye enlargement observed in the pnr mutant is due to ectopic induction of tsh in the dorsal eye, which in turn can induce the RD genes (Fig. 8).

Functional conservation of dorsal selector Pnr

The Drosophila eye is similar to the vertebrate eye in several features (Sanes and Zipursky, 2010) like: (i) the morphogenetic furrow in the fly eye is analogous to the wave of neurogenesis in the vertebrate eye (Neumann and Nuesselin-Volhard, 2000; Hartenstein and Reh, 2002), (ii) like Drosophila, in higher vertebrates dorsal eye genes like Bmp4 and Tbx5 act as “dorsal selectors” and restrict the expression of ventral eye genes Vax2 and Pax2 (Koshiba-Takeuchi et al., 2000; Peters and Cepko, 2002). These DV expression domains or developmental compartments (Peters, 2002) lead to formation of DV lineage restriction as seen in the Drosophila eye (Singe and Choi, 2003; Singh et al., 2005a), (iii) The DV lineage in the vertebrate eye also develops from a ventral-equivalent initial state (for review see Singh et al., 2005b). The dorsal genes pnr and iro-C are highly conserved across the species, and are involved in organogenesis and neural development (Gómez-Skarmeta and Modolell, 2002; Singh et al., 2005b). Therefore, it would be interesting to see whether the dorsal selectors in the vertebrate eye play a role in defining the boundary of the eye by suppressing retinal differentiation.

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subsequent Tukey’s HSD to determine significant differences for the data they have collected during this lab.

Conclusion

The above protocol offers instructors a dynamic teaching exercise that can be altered to accommodate students at a variety of levels, from an introductory genetics course, to an advanced behavioral or developmental genetics course. This teaching protocol gives students an opportunity to gain experience working with *Drosophila*, a model species widely used in the study of behavior, development, and genetics. The protocol provides students with hands-on experience in the acquisition of scientific data, from the basic level of sample collection and preparation, to the more advanced data analysis and presentation. The protocol also provides the opportunity for direct experience assaying for mutant phenotypes and in more involved versions the possibility of incorporating actual mutagenesis experiments with this behavioral assay.


*Drosophila* adult eye model to teach Scanning Electron Microscopy in an undergraduate cell biology laboratory.

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Abstract

We have devised an undergraduate laboratory exercise to study tissue morphology using fruit fly, *Drosophila melanogaster*, as the model organism. *Drosophila* can be reared in a cost effective manner in a short period of time. This experiment was a part of the undergraduate curriculum of the cell biology laboratory course aimed to demonstrate the use of Scanning Electron Microscopy (SEM) technique to study the morphology of adult eye of *Drosophila*. The adult eye of *Drosophila* is a compound eye, which comprises of 800 unit eyes, and serves as an excellent model for SEM studies. We used flies that were mutant for Lobe (L), eyeless (ey), and pannier (pnr) for our studies. The mutant flies exhibit different morphologies of the adult eye. We employed a modified protocol, which reduces sample preparation steps and makes it practically feasible to complete the protocol in
assigned time for the cell biology laboratory. The idea of this laboratory exercise is to: (a) familiarize students with the underlying principles of scanning electron microscopy and its application to diverse areas of research, (b) to enable students to sharpen their observation and quantitative microscopy skills, and (c) minimize the preparation time for the instructor.

Keywords: Drosophila melanogaster, eye, tissue morphology, Scanning Electron Microscopy (SEM), cell biology, undergraduate education.

Introduction

Research is an important component of habits of inquiry and learning in the undergraduate curriculum. A large array of laboratory courses has been developed for undergraduate students in order to expose them to techniques used in biomedical research. Interestingly, many new text books and accompanying supplementary materials provide exhaustive and detailed information through images and movies on diverse subject material studied using the Scanning Electron Microscopy (SEM) technique. Although animations and videos can provide an overall idea, it is important that students get a “hands-on” exposure to learn the techniques like how to use SEM to capture high resolution images. We devised a laboratory to introduce students to the SEM technique, its principle and applications, which will allow them to get a hands-on experience on the scanning electron microscope. Furthermore, this exercise can be finished in a single laboratory session with some preparation done prior to the demonstration to the students.

For this laboratory exercise, we chose to study the morphology of the well studied adult eye model of Drosophila. This model is highly versatile as in addition to studying the pattern and morphology of the normal flies, the variations in eye development can be easily demonstrated. There are several molecularly characterized mutants that directly or indirectly affect the morphology of the adult eye. Using the normal and mutant flies, we can demonstrate the limitations of conventional light microscopy in terms of resolution and magnification. This may help the students to appreciate (i) SEM has a much greater resolving power than light microscopes, (ii) SEM uses electromagnetic radiation instead of light, and (iii) SEM can obtain much higher magnifications of up to a million times.

SEM produces images by probing the specimen with a focused electron beam that is scanned across a rectangular area of the specimen (raster scanning). The electron beam of SEM is generated from a filament, which may be made up of various types of materials. The most common filament is made up of a loop of tungsten which functions as the cathode. A beam of electrons is produced at the top of the microscope by heating this metallic filament. The electron beam follows a vertical path through the column of the microscope and makes its way through electromagnetic lenses that focus and direct the beam on the sample. Electrons in the beam interact with the atoms constituting the sample material and are scattered back, producing the back scattered electrons or the secondary electrons. A detector collects the secondary or backscattered electrons, and converts them to a signal that is sent to a viewing screen similar to the one in an ordinary television, producing an image (Figure1). These signals contain detailed information about the sample's surface topography, composition and other properties such as electrical conductivity.

Vacuum is an essential requisite for SEM. If the sample is in a gas filled environment, the beam is unstable as gases could react with the electron source, causing it to burn out or result in ionization of beam. Alternatively, other molecules, which come from the sample or the microscope itself, may form compounds and condense on the sample and thereby reduce contrast and obscure details in the image.
Figure 1. Schematic flow diagram of a Scanning Electron Microscope. Electron microscopes use a particle beam of electrons to illuminate a specimen and create a highly-magnified image. The electrons from electron gun pass through anode, electromagnetic lenses, coils, detectors and strike the gold coated specimen placed on the stage. The electromagnetic lenses focus electron beam to a specific plane relative to the specimen and thereby forming the image. The secondary electrons generated because of electrons striking specimen surface are detected by secondary electron detector and converted into signal that is sent to a TV scanner.

Protocol

We have employed Drosophila melanogaster to study the morphology of the adult eye. Drosophila eye is a compound eye made up of 750-800 unit eyes referred to as ommatidia. We selected the flies that were mutant for genes involved in eye development (obtained from the Bloomington Stock Center, Indiana; http://flystocks.bio.indiana.edu/). The Bloomington Stock center is a repository of various fly strains and mutations, which are available upon request to the scientific community. We selected fly mutant strains for genes eyeless (ey) [ey^2 (BL 648), which shows complete loss of the eye field], Lobe (L) [L/Y/CyO (BL 319), a mutant which shows selective loss of the ventral eye], pannier (pnr) [pnr^x6/TM6B, (BL 6334)], which is an embryonic lethal mutation that can generate dorsal eye enlargement in genetic mosaics where pnr function is eliminated in patches of cells (Xue and Rubin, 1993; for review see, Blair, 2003). These three different mutants exhibit a range of phenotypes of eye size from enlarged eye to half eye, and to no-eye in comparison to the wild-type eye (Figure 2). However, each of these mutants show a range of phenotypes due to penetrance. Therefore, for our lab exercise, we selected flies that showed distinct eye phenotypes from a large population of each mutant stock.

This exercise helped students to learn two basic experimental operations: (a) sample preparation, and (b) basic operation of the scanning electron microscope. Students also learned some background information on the development and morphology of the normal eye.

The entire methodology of the SEM can be divided into three major steps: (1) sample preparation, (2) sample mounting and sputter coating, and (3) imaging.

1. Sample Preparation:

This step includes preparation of sample and is carried out prior to the research laboratory. For SEM, biological samples need to be dehydrated and dried. Dehydration is carried out to
gradually reduce the water content of the tissue to the point that the tissue is completely into a non-aqueous solvent. Dehydration is done using ascending concentration series of ethanol or acetone. The flies of different eye mutants were passed through a series of ascending concentrations of acetone to dehydrate the sample. The adult fly samples were dehydrated by incubating for 24 hours each in 30%, 50%, 70%, 90%, 95%, and 100% concentrations of acetone. Thus at the end of the seventh day, the sample is completely dehydrated and is present in 100% acetone. To achieve best results, sample was dehydrated in 100% acetone twice.

In earlier protocols, dehydrated samples were subjected to critical point drying. The presence of surface tension during drying is disruptive to tissues and causes visible distortions. Therefore, the critical point drying is carried out in vacuum where fluid and gaseous phases exist together and there is no surface tension. The critical point drying is achieved using liquid carbon dioxide (CO₂). However, drying can also be achieved using commercially available chemicals. Here, we employed Hexamethyl Di Silazane (HMDS), which is a chemical of choice used for drying SEM samples that mainly include insect tissues, large fleshy tissues or soft invertebrates (Braet et al., 1997). Furthermore, it does not require vacuum. Drying with HMDS prevents the tissue morphology from getting damaged in freeze drying or liquid CO₂ drying procedures. Following 100% acetone, samples were incubated overnight in 1:1 mixture of 100% acetone: HMDS (Electron Microscopy Sciences Cat# 16700). Samples were then incubated in 100% HMDS solution for 24 hours and they were allowed to air dry at room temperature in a fume hood. The lids of the tubes were left open to allow the HMDS to evaporate.

2. Sample Mounting and Sputter Coating:

Each HMDS treated dehydrated sample was mounted on a metallic stub (a sample holder for electron microscope, available from Electron Microscopy Sciences Cat# 75944). Sample was held onto the stub by a conductive carbon tape (Electron Microscopy Sciences Cat# 77825-12). Maximum contact of the sample with the tape was ensured so that sputter coating is good. Sample was arranged on the stub in such a way that the area of interest (in this exercise the eye tissue) in the sample is perpendicular to the plane of the observation in the microscope. Mounted tissue on the stub was then sputter coated in vacuum with an electrically conductive layer of gold (or some other inert heavy metal). This step is important since it makes the sample conductive, enhances the secondary and backscattered electron emission and increases the mechanical stability of the tissues. Coating is an essential step to prevent accumulation of static electric charge on the specimen during electromagnetic irradiation. Improper coating on the tissue results in charging, which may result in deflection of electron beam, deflection of secondary electrons and periodic burst of secondary electrons. Gold is the preferred metal for coating the samples because of its high atomic number. Further, sputter coating with gold produces high topographic contrast and resolution. Depending on the type of sample, there are several other coating materials like Gold Palladium alloy, Platinum, Iridium, Tungsten, Osmium, Graphite and Carbon. The sample stub was subjected to sputter coating at pressure of 100 psi for a period of 35 seconds and a current of 45 milli amps under vacuum using sputter coater (DV 502) from Denton Vacuum Company.

We have described a protocol for sample preparation for SEM. However, there have been continuous improvements in the processes of sample fixing, drying and coating methods. A variety of new adaptations to SEM have also emerged that enables a large spectrum of samples to be analyzed using SEM technique. There are alternative methods for fixation, dehydration and coating depending on the nature of sample and approach used summarized in Table 1.
Table 1. Alternative materials used in SEM for fixation, dehydration, drying and sputter coating in different model systems.

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>References</th>
<th>Fixative</th>
<th>Dehydrating agent</th>
<th>Drying process</th>
<th>Mounting/ Sputter coating</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteria, virus on surfaces, as parasites</td>
<td>Sangetha et al., 2009; Robinson et al., 1984</td>
<td>Glutaraldehyde, Osmium tetra Oxide (OsO₄)</td>
<td>Ethanol</td>
<td>Critical point drying in amyl acetate</td>
<td>Gold, Gold-Palladium alloy</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plant tissues</td>
<td>Pathan et al., 2008</td>
<td>Glutaraldehyde, Osmium tetra Oxide (OsO₄)</td>
<td>Ethanol/ Acetone</td>
<td>Critical point drying</td>
<td>Mounting using Ag. Silver, Coating with chromium</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Insect tissues</td>
<td>Braet et al., 1977, (modified by Naoto Ito)</td>
<td>Fixing is usually not required</td>
<td>Acetone</td>
<td>HMDS (Chemical drying)</td>
<td>Gold</td>
</tr>
<tr>
<td>Mammalian tissue</td>
<td>Wierzchos et al., 2008; Lehman et al., 1983</td>
<td>Aldehyde/ Formalin/ Osmium tetra Oxide (OsO₄)</td>
<td>Ethanol</td>
<td>Critical point drying in acetone</td>
<td>Gold, Gold-palladium alloy, Carbon coating</td>
</tr>
</tbody>
</table>

3. Imaging:

The final step in this exercise is to image the samples using SEM. Sputter coated sample stub was then imaged using the Hitachi S-4800 High Resolution Scanning Electron Microscope (HRSEM) available in the Nanoscale Engineering Science and Technology (NEST) facility at University of Dayton. The samples on the stub were placed in vacuum and subjected to electron beam. A voltage of 5kV was applied. The electrons from the gun strike the surface coating of gold, electrons are reflected back off the specimen to a detector, this is transmitted to a TV screen where the image is viewed and photographed. The images were taken at 130×. At magnification of 130×, the entire *Drosophila* head fits in an image plane and is a suitable resolution to study morphology of head and the compound eye. As shown in the Figure 2, each unit eye or the ommatidium is clearly visible, and this would not have been possible using a compound light microscope of 10× magnification. The high resolution SEM images provides detailed information about different kinds of bristles present among the ommatidia. Depending on the model, SEM allows the magnification of a sample up to 500,000-1000000 times.

Advantages

1. The greatest challenge to teaching a Cell Biology laboratory is the capital investment/commitment that a university/college must make to laboratory. The use of cost effective exercises can facilitate the execution and implementation of these laboratory programs in an undergraduate academic institution setup.

2. The students get general overview of SEM and hands-on experience of the technique starting from sample preparation to visualizing the sample on the monitor attached to SEM.

3. The sample preparation in conventional method for SEM is a little time consuming procedure. It requires critical point drying in vacuum. In our protocol, we eliminated the critical point drying method which requires vacuum.
4. Our protocol does not require post fixation treatment with Osmium tetra Oxide (OsO₄), which is highly carcinogenic and may not be an ideal chemical to use in an undergraduate laboratory. Instead, we use HMDS for final processing after dehydration in acetone series.

5. These exercises does not require educational demonstration kits that minimize the exposure of experimental details and reagents to the students.

6. This experience adds to their skill-set and helps generate a core of trained individuals who can function in academics as well as corporate settings.

High magnification images are powerful sources of communication which are preferred to words. Most laboratory science courses do not actively teach students skills to communicate effectively through images (Riemeier and Gropengießer, 2007). Our laboratory exercise meets this requirement by teaching students to (a) develop basic laboratory skills and learn tissue handling,
sample preparation and scanning electron microscopy, (b) capture digital images using the software,
(c) process the image using the Photoshop or imaging software, and (d) develop a series of image
portfolios to present their results.

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1237.

Heat shock effects upon cell death in Bar eye quantified by scanning electron microscopy.

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Bar (B) is a well-known sex-linked dominant mutation that arose spontaneously in
Drosophila melanogaster as a tandem duplication in cytological location 16A1-2 (Tice, 1914;
Lindsley and Zimm, 1992). The vertical bar-eye phenotype is due to cell death, especially in the
anterior region of the eye disc (Fristrom, 1969), or disruptions in the pattern of mitosis. But the
extent of cell death can be influenced genetically (e.g., variegated position effect; Brosseau, 1960)
and by environmental conditions like temperature (e.g., developmental temperature and log facet
number are inversely proportional; Hersh, 1930) and chemical treatments (e.g., being raised on media
supplemented with acetamide, lactamide, cytosine, and other chemicals; Fristrom, 1972; and
references in Lindsley and Zimm, 1992). Given its sensitivity to modifying factors, the severity of
Bar eye cell death can be a model for quantifying experimental influences on development. But for
this model system to be sensitive enough to detect comparatively small effects, eye facet
(ommatidium) number must be measured very accurately. In spring 2009, the Experimental Genetics
and Cell Biology Lab course taught in the Department of Zoology at the University of Oklahoma
undertook to test experimental design options and the feasibility of using scanning electron
microscopy of Drosophila Bar eyes to evaluate the effect on cell death by an experimental treatment,
exposure to heat shock that activates chaperone proteins of the stress response. Additional data were
Teaching List of Contributions


http://www.ou.edu/journals/dis/teachingarticles.html
A cell biology laboratory exercise to study sub-cellular organelles in *Drosophila*.

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Key words: *Drosophila melanogaster*, cell biology, sub-cellular organization, Immunohistochemistry, undergraduate education

**Abstract**

The fast changing scenario of undergraduate education puts emphasis on introducing students to hands-on techniques as part of their laboratory courses. In order to cater to large numbers of students and the time constraints involved with undergraduate level laboratory courses, there is a need for development of experiments that are cost effective and can be completed in a defined time frame. We have devised a laboratory exercise for teaching cell biology using the *Drosophila melanogaster* model. *Drosophila* can be reared in a short period of time in a cost effective manner. We used *Drosophila* tissue to study the sub-cellular organization of eukaryotic cells using fluorescent markers. The idea of this lab exercise is to: (a) familiarize students with the underlying principles of cell structure and function and its application to diverse areas of research, (b) allow students to sharpen their observation and quantitative microscopy skills, and (c) minimize the preparation time for the instructor.

The participation of undergraduates in research and experimental learning is an important component of several undergraduate programs. One of the important facets of a cell biology course for an undergraduate curriculum is to introduce the structural and functional aspects of the various sub-cellular organelles. Interestingly, many new text books and accompanying supplementary material provide exhaustive and detailed information, through images and movies. Although animations and videos can elucidate the complex organization of organelles in the cell, it is important that students also have the opportunity for laboratory experiences. In order to introduce undergraduate students to the sub-cellular organelles in a eukaryotic cell, we have started developing laboratory exercise(s) which can be performed in an undergraduate classroom setup.

The cell is the basic structural and functional unit of life. The sub-cellular organization of the eukaryotic cells includes different membrane bound organelles, which have specialized structures and functions. For example, the nucleus contains the genetic information in the form of DNA and RNA, the Golgi complex and the endoplasmic reticulum are specialized for packaging and folding of proteins, lysosomes contain the hydrolytic enzymes, microtubules or the microfilaments are required to provide support to the cells. Thus, different organelles are specialized for performing different functions. Any defect in their structures leads to impairment of their respective functions which eventually may lead to cell death, or have deleterious consequences on cell/organelle or organ function leading to diseases. Therefore, it is useful to observe these organelles at high resolution under the microscope, in order to understand their structures and functions.

In the majority of cell biology laboratories, students are shown the sub-cellular organelles either through images, movies, or from permanent commercial slides. However, appreciation for the different protocols/techniques used to stain cells with traditional or fluorescent dyes is lost upon the
students mostly, because they do not understand the underlying principles of organelle specific staining in cells. Therefore, we decided to develop a laboratory exercise to observe various organelles in fixed Drosophila tissues by using dyes in an undergraduate laboratory setup.

Different sub-cellular organelles were marked using a set of commercially available fluorescent molecules that bind specific biochemical compounds in the cells which are localized to a particular organelle (Lavis et al., 2006). These markers, thus, tag different organelles, which can then be observed using an epifluorescence microscope. Each marker can be visualized based on its excitation/emission properties. A variety of such molecules are commercially available (http://www.invitrogen.com/site/us/en/home/brands/Molecular-Probes.html) such as Lyso Tracker for marking lysosomes, Mito Tracker for mitochondria, Phalloidin (Small et al., 1999) which marks actin filaments, and DAPI (4'-6-Diamidino-2-phenylindole) (Kapuscinski, 1995) which tags DNA in the nucleus (for details, see Table 1).

We used fluorescent dyes that act as markers for the actin filaments and the nuclei in a eukaryotic cell. The undergraduate students worked in pairs and performed this experiment as a part of cell biology laboratory course. The entire experiment lasted less than three hours which involved dissection, tissue fixation, incubation, and observation under the microscope.

Table 1. List of dyes that can be used to mark the organelles in the cell.

<table>
<thead>
<tr>
<th>Organelle</th>
<th>Dye</th>
<th>Molecular Probes Catalogue number</th>
<th>Excitation (nm)</th>
<th>Emission (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mitochondria</td>
<td>Mito Tracker Green (Vanden Berghe, 2004)</td>
<td>M22425</td>
<td>644</td>
<td>588</td>
</tr>
<tr>
<td></td>
<td>Mito fluor Green probe</td>
<td>M 7502</td>
<td>588</td>
<td>622</td>
</tr>
<tr>
<td></td>
<td>Mito Sox red</td>
<td>M36008</td>
<td>510</td>
<td>580</td>
</tr>
<tr>
<td>Lysosomes</td>
<td>Lysotracker</td>
<td>L7528</td>
<td>577</td>
<td>590</td>
</tr>
<tr>
<td>Golgi Complex</td>
<td>NBD C$_{2}$-Ceramide</td>
<td>N-1154</td>
<td>466</td>
<td>536</td>
</tr>
<tr>
<td>Endoplasmic Reticulum</td>
<td>Bodipy (Lavis et al, 2006)</td>
<td>D7540</td>
<td>589</td>
<td>617</td>
</tr>
<tr>
<td>Cytoskeleton</td>
<td>Texas Red Phalloidin (Small et al, 1999)</td>
<td>T7471</td>
<td>591</td>
<td>608</td>
</tr>
<tr>
<td></td>
<td>DAPI (Kapuscinski, 2006)</td>
<td>D 1306</td>
<td>358</td>
<td>461</td>
</tr>
<tr>
<td>Nucleus</td>
<td>TOPRO</td>
<td>T7596</td>
<td>745</td>
<td>770</td>
</tr>
<tr>
<td></td>
<td>Hoescht</td>
<td>H21486</td>
<td>350</td>
<td>450</td>
</tr>
</tbody>
</table>


Protocol

Flies were cultured on yeast-cornmeal-agar fly medium (recipe available at http://flystocks.bio.indiana.edu/Fly_Work/media-recipes/molassesfood.html) at room temperature and allowed to lay eggs. Third instar larvae (wandering on the wall of the culture vial) were dissected in PBS (Phosphate Buffered Saline, containing 137 mM NaCl, 10 mM Phosphate, 2.7 mM KCl, pH 7.4; Dulbecco et al., 1954) using sharp Dumostar forceps (Electron Microscopy Sciences (EMS) Cat. No. #72707-01). The salivary glands and imaginal discs attached with mouth parts and brain were dissected. Imaginal discs are groups of epithelial cells housed inside the larva that will give rise to adult appendages and the cuticle after metamorphosis.

Tissue was fixed in 4% paraformaldehyde (EMS Cat. No. #15710) in PBS to preserve the morphology. The fixative was removed after fixing the tissue for twenty minutes. The tissue was
rinsed in ice cold PBS, followed by washes with PBST [PBS+ 0.2% Triton X-100 (Sigma Aldrich Cat No. T100)], to permeabilize the tissue. The tissue was then incubated with Texas Red Phalloidin- (Molecular Probes, Invitrogen, Cat. No. # T7471) and DAPI (Molecular Probes, Invitrogen, Cat. No. # D 1306) in PBST for twenty minutes at room temperature in dark. The phalloidin specifically marks the actin filament meshwork of the salivary gland cells (Figure 1a, b; red channel). The DAPI stains the nuclear material within the cell of the salivary gland (Figure 1a, b; green channel). In salivary glands, endoreduplication causes polyploid DNA that can be easily visualized in the large nuclei of salivary gland cells. The washing steps were performed using PBST and finally the tissue was mounted on glass slides in Vectashield mountant (Vector labs, Cat. No. # H-1000). Vectashield serves as an antifade agent that helps to preserve fluorescence in stained tissue.

The tissue was then observed under a Carl Zeiss epifluorescence microscope (Axio Imager. Z1). Phalloidin was observed in Alexafluor 546 channel as it has an excitation wavelength of 494nm and emission wavelength of 517nm. DAPI was observed under DAPI filter (Table 1). The digital images were obtained using CZ Focus software on the Zeiss Apotome epifluorescence microscope or Olympus Fluoview 1000 confocal microscope. Images were processed using Adobe Photoshop 5.5 software. Alternatively, images can be taken on a conventional epifluorescence microscope camera with a high speed film.

![Figure 1](image_url)

**Figure 1.** Labeling eukaryotic cell /organelle in *Drosophila* tissue using fluorescent dyes. (a) *Drosophila* salivary gland stained for DAPI (Green) that marks the nuclei and Phalloidin (Red) which marks the actin filaments. (b) Magnified view (63×) of the salivary gland cell.

**Advantages**

1. The greatest challenge to teaching a cell biology laboratory is the capital investment/commitment that a university/college must make to laboratory. The use of cost-effective exercises can facilitate the execution and implementation of these laboratory programs in undergraduate academic institution setup.

2. The students were able to get hands-on experience of the technique and visualize the results in a fluorescent microscope in a single teaching laboratory credit hour of three hours per week.
3. These exercises did away with the use of tailor-made demonstration kits provided by companies.

4. This laboratory exercise helped in generating permanent slides, which can be used for final exams where students can be asked to identify the organelle. This will prevent the expense of buying commercial slides for exams.

5. It will help generate a core of trained individuals who can function in academic as well as corporate settings.

Conclusion

Cell biologists rely on images to communicate their results and to study/teach structure and function of cells. Images are powerful means of communicating scientific results. A clear high-magnification image can underscore an experimental result more effectively than any words, whereas a poor image can readily undermine a result or conclusion (Pearson, 2005; Chatterjee, 2006). Most undergraduate laboratory science courses do not actively teach students skills to communicate effectively through images (Riemeier and Gropengießer, 2007). Our laboratory exercise meets this need by teaching students to (a) develop basic laboratory skills and learn immunohistochemistry and fluorescence microscopy, (b) capture digital images using the software, (c) process the image using the Photoshop or imaging software, and (d) develop a series of image portfolios to present their results.

Additional Resources

There are several resources available at Bloomington Stock Center (http://flystocks.bio.indiana.edu/), which can be used for cell biology laboratory in undergraduate institutions. Some of the easy to work reagents are:

a. Protein-trap lines: Transgenic flies, where the proteins are tagged with the GFP reporter and these proteins localize specifically to some organelles in the cell (http://flytrap.med.yale.edu/index.html).

b. Reporter lines: There are transgenic flies available that specifically mark organelles like Golgi (BL-7193), Mitochondrion (BL-7194), and Endoplasmic reticulum (BL-7195), by the presence of EYFP reporter. These stocks can be used to demonstrate sub-cellular organelles in the cells in a cost effective and time efficient manner.

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A concise *Drosophila* laboratory module to introduce the central concepts of genetics.

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**Overview**

The quick generation time, ease of handling, and wide availability of striking phenotypic mutants makes *Drosophila melanogaster* a highly attractive system to expose undergraduate and advanced high school students to concepts in genetics. However, it is nevertheless very labor intensive to set up *Drosophila* for a large scale laboratory exercise in a short period of time. The previously developed P{his-hid}Y heat shock system enables a greatly optimized procedure for the collection of virgin females (Venema, 2006; Venema, 2008). Using this system allowed us to develop a short laboratory module that can be completed in only three 2-3 hour long laboratory sessions spaced two weeks apart. This format also permits the *Drosophila* genetics module to be interspersed with other modules in a single semester laboratory course.

This introductory *Drosophila* genetics laboratory module emphasizes hypothesis-driven scientific inquiry by encouraging students to form their own open-ended questions about the nature of mutations and the pattern in which they are inherited from one generation to the next. The two crosses in the module introduce several important genetics concepts including: Mendelian autosomal inheritance, sex-linked inheritance, recombination, genetic mapping, and non-disjunction. Students are also introduced to the method of using statistical tests to validate or reject biological hypotheses.

**Methods**

The P{hs-hid}Y stocks (Venema, 2006) greatly facilitate setting up and collecting virgin female *Drosophila* for genetic crosses. The P{hs-hid}Y line has a P element insertion on the Y chromosome containing a proapoptotic lethality gene, *head involution defect (hid)*, driven by the Hsp70 heat shock promoter (Grether *et al*., 1995; Starz-Gaiano *et al*., 2001). Heat shocking flies at mid-larval stages for 2 hours activates expression of *hid*, causing the death of all male (Y chromosome-carrying) larvae (Figure 1A and B). Consequently, only the females survive to become adult flies. The P{his-hid}Y system can be applied to any mutant line by crossing P{his-hid}Y males with virgin females homozygous for the mutation of choice and then backcrossing the F1 males with the mutant phenotype to the original stock of homozygous mutant virgin females to create a stable P{hs-hid}Y line with the mutant phenotype.