Amit Singh
Madhuri Kango-Singh *Editors*

Molecular Genetics of Axial Patterning, Growth and Disease in Drosophila Eye

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Editors
Amit Singh
Department of Biology
University of Dayton
Dayton, OH, USA

Madhuri Kango-Singh
Department of Biology Office: SC303C
University of Dayton
Dayton, OH, USA

ISBN 978-3-030-42245-5 ISBN 978-3-030-42246-2 (eBook)
https://doi.org/10.1007/978-3-030-42246-2

This Springer imprint is published by the registered company Springer Nature Switzerland AG
The registered company address is: Gewerbestrasse 11, 6330 Cham, Switzerland

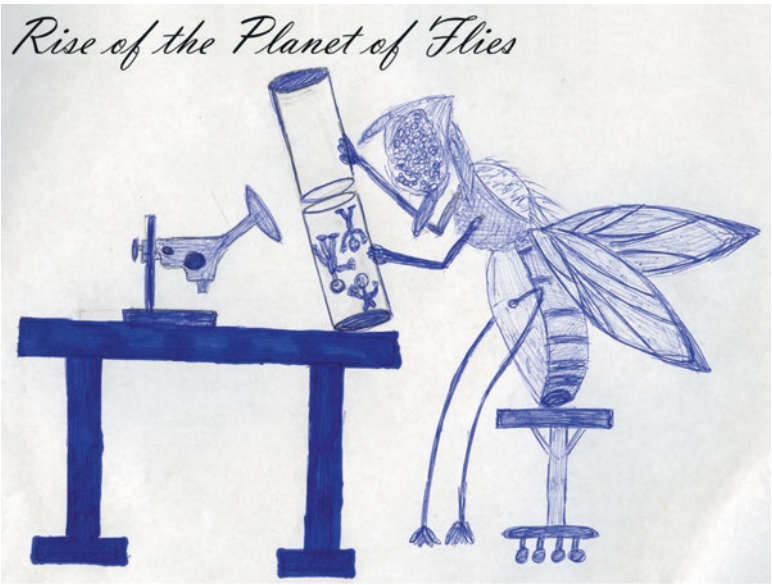
To
Our Parents
Late Prof. Hari Om Singh
Mrs. Dinesh Kumari Singh
Late Mr. D.M. Kango
Prof. Mangala Kango &
Our Daughters
Aditi Singh
Manasi Singh

Introduction

The fly sat upon the axle tree of the chariot-wheel and said, what a dust do I raise!—Aesop

The quest to understand how a single-celled embryo is transformed into a multicellular three-dimensional organism with complex structure and functions has been a challenge for the developmental biologists for ages. This question resembles the search for the holy grail of modern-day biology. During the development of a multicellular organism, cell proliferation is tightly regulated to produce specific number of cells, which in turn is followed by a fundamental process of differentiation that is regulated by a genetic circuitry. Any perturbation in this finely tuned process results in defects. Therefore, the basic cell biological process of cell proliferation, cell differentiation, and cell death play important roles in sculpting an organ during organogenesis. Since the genetic machinery is highly conserved, it has been pointed out that the basic core machinery involved in regulating these fundamental processes are similar. In developmental biology, it is important to unravel the mechanism of fate assignment and differentiation.

The time tested *Drosophila melanogaster* (fruit fly) model has played a central role in developmental biology during the twentieth century. The *Drosophila* model has a long genetic legacy, beginning with Thomas Hunt Morgan in early 1900 (Morgan 1911). A judicious blend of molecular and developmental genetics has proved beyond doubt that *Drosophila* is a valuable model for addressing important questions of modern-day biology. There are several thousand people whose work/lives center around the little fruit fly *Drosophila melanogaster*. In recent years, the emphasis of their studies has shifted from inheritance to development and disease. In the hands of a small number of particularly imaginative scientists, traditional genetics, experimental embryology, and new molecular genetic techniques have been combined to build a picture of developmental mechanisms. To date, *Drosophila* has maintained its status as a trusted and highly versatile model to study patterning, growth, and disease. Among all the adult body structures, the *Drosophila* eye, because of its simple structure and easy amenability to mutations and genome-wide screens has become an important tool in the hands of Drosophilists.



The study of developing eye from a two-dimensional eye primordium to a three-dimensional adult eye and visual system, and the use of eye model to study patterning, growth, development, evolution, and disease is the topic of the current book. The *Drosophila* eye has been intensively studied to explore cell biological processes like cell fate specification, patterning, growth, and cell signaling. Understanding the generation and functioning of eye as an organ, our primary sensory modality, is important. We are curious to know how the visual system assembles.

It is now almost 37 years since the seminal paper from Ready et al. (1976) described the development and structure of *Drosophila* compound eye. The discovery of morphogenetic furrow (MF), a wave of differentiation, which is initiated from the posterior margin of the eye imaginal disc and sweeps in the anterior direction (Ready et al. 1976), is considered to be a major milestone in *Drosophila* eye field. It results in differentiation of retinal precursor cells to photoreceptor neurons. It was known that adult appendage develops from a group of cells set aside during embryonic development, which grows during larval stages and then metamorphose into adult appendages. Tomlinson provided the electron microscopic view of cellular events that follow the formation of morphogenetic furrow (Tomlinson 1985). Generation of monoclonal antibodies to detect early cell differentiation was another major landmark (Fujita et al. 1982). Enhancer trap technique using P element-mediated transgenesis proved to be an important tool that still remains an asset in the arsenal of modern-day fly geneticist's tool kit (Bellen et al. 1989; Grossniklaus et al. 1989; Wilson et al. 1989). Another important milestone was demonstration of structural and functional similarity in the genetic circuitry involved in eye development in flies and humans (Halder et al. 1995; Quiring et al. 1994). These studies completely changed the outlook of the eye field. Halder et al. (1995) reported the

master selector gene concept in the eye where they demonstrated that *eyeless (ey) Drosophila* homolog of PAX-6 gene could reprogram other tissues and generate ectopic eyes in the wing, leg, and antenna. These studies provided a great impetus to the *Drosophila* eye model, which by then was also used to address questions for human disease. The evolution of *Drosophila* eye research cannot be complete without mentioning the contributions of Seymour Benzer, Walter Gehring, and Gerald M. Rubin. The hard work of Gerald Rubin and his collaborators came to fruition when fly genome was published in 2000 (Adams et al. 2000; Myers et al. 2000; Rubin et al. 2000). It was instrumental in validating the observation of Gehring's group that there is a strong conservation in the genetic circuitry of flies with that of humans and other vertebrates. It completely changed the field and put the fly model on the forefront among all other animal models. These discoveries led to generation of new genetic and molecular technology, and put *Drosophila* eye model system on the forefront of biological research to address important questions related to human diseases like retinal diseases, neurodegenerative disorders, and cancers. Furthermore, the *Drosophila* eye model provided more versatility to study basic cell biological processes of patterning, growth, cell proliferation, and cell death and to carry out genome-wide screens.

This picture is new and exciting although it is far from complete. It represents the beginnings of a real understanding of how one animal is designed and built. This book, which is written for the students as well as the specialists, aims to give an up-to-date glimpse of that picture. However, the field is developing so fast that some of the things may change; therefore, we have tried to use well-established material. We have made an attempt to provide an overview of approaches used in the fly eye model. We have dealt with the basic question of patterning of how eye develops starting from early events of specification to molecular mechanisms involved in transition of eye from a monolayer epithelium to a three-dimensional structure. During this transition, one of the hallmark events is formation of the morphogenetic furrow (MF). This book also highlights events of morphogenesis, cell polarity, cell adhesion, and negative regulation of neural patterning in developing *Drosophila* eye. Other areas discussed in this book are the use of Drosophila eye model to understand protein homeostasis network, organ size control mechanism, and genetic basis of neurodegeneration. The book also encompasses an important aspect of development and evolution during early eye development as well as larval eye or Bolwig's organ.

The collection of chapters in this book helps us celebrate hundred plus years of research using *Drosophila* eye model and provides a blueprint of future research directions and frontiers in this field. We hope you enjoy reading this book as much as we did. We would like to end with a quotation (Dryden J (1696) from: *The epilogue to The Husband his own Cuckold*, lines 35–37):

> Fools change in England, and new fools arise'
> For, tho' th' immortal species never dies,
> Yet ev'ry year new maggots make new flies …..'

Acknowledgements We would also like to extend our gratitude to the fly community and their support for this venture. There is not enough space to mention the names of all the researchers whose contribution in the *Drosophila* eye field has been instrumental in making this book a reality. The *Drosophila* eye model has been exceptionally lucky to get attention from a strong group of highly accomplished scientists. It has been a great pleasure for us to work or interact with many of them and to hear them in the meetings. We would like to thank the fly pushers who have been kind enough to contribute to this book.

We are grateful to our mentors Henry Sun, Kwang-Wook Choi, Georg Halder K.P. Gopinathan, and Pradip Sinha for introducing us to this "Golden Bug" or "Cinderella of Modern Genetics" and to the field of patterning and growth in the developing eye and other organs.

This book would not have been possible without the excellent support from Editors at Springer who worked patiently and diligently to help keep the process of writing streamlined and manageable.

The encouragement and support for initiating this project was provided by my mother Dinesh Kumari Singh. We would like to thank our daughters Aditi and Manasi and brother Rohit Singh who never doubted our ability even though they could not believe that any sane person can be fascinated by flies to this extent. Their perception of the fly is enclosed in figure.

Undoubtedly, Drosophila melanogaster, fruit fly, has proven to be one of the most popular invertebrate model organisms, and the workhorse for modern-day biologists. Drosophila, a highly versatile model with a genetic legacy of more than a century, provides powerful genetic, cellular, biochemical, and molecular biology tools to address many questions extending from basic biology to human diseases. One of the most important questions in biology focuses on: how does a multicellular organism develop from a single-celled embryo? The discovery of the genes responsible for pattern formation has helped refine this question. Drosophila eye model has been extensively used to study molecular genetic mechanisms involved in patterning and growth. Since the genetic machinery involved in the Drosophila eye is similar to humans, it has been used to model human diseases and homology to eyes in other taxa. This book will discuss molecular genetic mechanisms of pattern formation, axial patterning, growth regulation in Drosophila eye, and more.

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Contents

Early Eye Development: Specification and Determination

Abhishek K. Mishra and Simon G. Sprecher

Introduction

The visual system is required by animals to perceive, process, and transform visual information in order to build an internal representation of the visual environment. Even though eyes have evolved several times independently, many key features in the underlying organization as well as genetic and molecular mechanisms of eye development are shared in distinct animal clades. Although there are fundamental anatomical differences between compound eye of insects and vertebrate lens eye, the basic mechanism that regulates development of the visual system seems to be conserved throughout evolution (Quiring et al. 1994; Desplan 1997; Neumann and Nuesslein-Volhard 2000; Brown et al. 2001; Kumar 2001; Pappu and Mardon 2002). The insect eye contains large array of hexagonal-like unit eyes called ommatidia. The number of ommatidia per eye varies largely between different insect species, and it mainly depends on the size of the eye. For example, each compound eye of the adult fruit fly *Drosophila melanogaster* consists of approximately 800 ommatidia, which forms a highly stereotypically organized neurocrystalline lattice (Ready et al. 1976). Each ommatidium consists of a core of 8 light-sensing neural cells surrounded by 12 supporting nonneural cells. The neural cells, also called photoreceptor (PR) neurons, are highly specialized photosensitive cells that transmit visual inputs inside the brain. At the distal end of each ommatidium, there are four cone cells which sit above the PRs and secrete corneal lens and pseudocone. The two

A. K. Mishra
Department of Biology and Department of Cell Biology, University of Virginia, Charlottesville, VA, USA

Department of Biology, University of Fribourg, Fribourg, Switzerland

S. G. Sprecher (✉)
Department of Biology, University of Fribourg, Fribourg, Switzerland
e-mail: simon.sprecher@unifr.ch

A. Singh, M. Kango-Singh (eds.), *Molecular Genetics of Axial Patterning, Growth and Disease in Drosophila Eye*,
https://doi.org/10.1007/978-3-030-42246-2_1

primary pigment cells together with six secondary and tertiary pigment cells encircle the PRs to limit light scattering (Cagan and Ready 1989a; Wolff and Ready 1993; Charlton-Perkins and Cook 2010). Each compound eye originates from a monolayer epithelium in the larva called eye-antennal imaginal disc. The posterior part of the disc that is designated as eye disc gives rise to all neural and nonneural cell types of the eye as well as the vertex during late larval to pupal stages. Fatemap studies have revealed that disc precursors are specified in the embryo that proliferates while the animal grows via three larval stages. At the second larval instar, anterior part of the disc which give rise to antenna becomes morphologically distinct from the posterior part that later forms retina and additional head cuticles. At the end of third instar larval stage, epithelial-to-neuronal transition occurs in the eye disc resulting in the initiation of retinal differentiation from posterior to anterior end as a wave. This differentiation wave is called morphogenetic furrow (MF). The MF moves from posterior to anterior end of the eye disc that results in the patterning of proliferating and undifferentiated cells into highly organized clusters called ommatidial clusters (Wolff and Ready 1993). Once the entire eye field is established, terminal differentiation occurs which are marked by removal of additional cells by apoptosis, synthesis of visual pigments, and formation of PR cell rhabdomeres that are light-sensitive microvillar structures. After the completion of terminal differentiation, two-thirds of the posterior eye disc transform and become retina, while the anterior third develops as head cuticle (Haynie and Bryant 1986).

While the adult imago only emerges after metamorphosis, the specification of eye precursors start early during embryogenesis. There are mechanisms that prevent initiation of adult retinal differentiation in the embryo, maintain growth and proliferation of eye field during postembryonic larval stages, and coordinate adult retinal differentiation with the complex process of metamorphosis. The eye disc is specified in the embryo and larval stages by a gene regulatory network called retinal determination network (RDN). The RDN includes *eyeless* (*ey*) which is often referred to as "master control gene of eye morphogenesis." The first *ey* mutant in *Drosophila* was described more than 100 years ago (Hoge 1915) and subsequently mapped to the fourth chromosome of the fly. However, important insights into the functional complexity of this gene start much later around 25 years ago when *ey* was first cloned and sequenced (Quiring et al. 1994). It leads to the astonishing observation that this gene belongs to *Pax6* family of transcription factor known to cause aniridia in humans and *small eyes* in mice (Hill et al. 1991; Ton et al. 1991; Walther and Gruss 1991; Quiring et al. 1994). Misexpression of *ey* in other imaginal discs hijacks the developmental program and transforms them as retina resulting in ectopic eyes such as in the antenna, wings, or legs (Halder et al. 1995). However, expression of *ey* does not always correspond to the formation of eyes. For example, *ey* is also expressed in the embryonic central nervous system but does not transform them into eyes. Therefore, it will be interesting to know those additional factors that allow *ey* to induce ectopic eyes in specific tissues. Based on its sufficiency for eye development, *ey* was proposed as a master regulator whose transient burst even could initiate retinal development (Gehring 1996). This discovery has challenged the view of the evolutionary relationship of different eyes across species; since both

Drosophila and vertebrate *Pax6* share the same function, the theory of monophyletic origin of the eye has been evolved (Halder et al. 1995; Gehring 2002). Apart from *ey*, other members of the RDN in *Drosophila* also has its vertebrate counterparts implicating that although camera-type eyes of vertebrates and compound eyes of insects are morphologically different, molecular mechanisms governing the eye development are surprisingly conserved within species. This discovery has made visual system in *Drosophila* as an excellent model system to understand development of vertebrate eyes and to analyze and model human ocular disorders into fruit flies.

In this chapter, we will review early eye specification and determination by first summarizing the knowledge gained so far about each member of RDN and how their genetic interactions guide early eye specification process. In the next section, we will discuss development of eye precursors in the embryo and how eye field is established in the developing eye-antennal imaginal disc during different larval instar stages. In the last section, we will summarize details about how retinal determination genes control extraretinal PR development in *Drosophila* that includes larval eye and adult ocelli.

The Retinal Determination Network (RDN)

The commitment of producing retinal fate from a population of uncommitted cells is called retinal determination. Over the past decades, several transcription factors have been found to mediate this process by forming a network called retinal determination network (RDN). In *Drosophila*, RDN initiates a process during which undifferentiated cells are specified and incorporated into the ommatidial structure of the adult retina. Genes in this network perform multiple tasks to coordinate cell proliferation, regulate initiation and migration of the MF, maintain individual cell fates, and eliminate excessive cells by apoptosis. To deliver multiple functions, members of RDN are involved in several reinforcing positive feedback loops, mutual negative interactions, and self-fortifying autoregulatory feedback mechanism (Kumar 2009a, b). They integrate multiple signaling pathways into the RDN at multiple levels, and these signaling pathways regulate transcription of individual genes in the network (Chen et al. 1999; Kurata et al. 2000; Kumar and Moses 2001a; Kenyon et al. 2003). The network begins during eye field determination in the embryo when retinal precursor cells are set aside to adopt an eye fate (Cohen 1993; Held 2002). RDN then initiate the formation of MF and controls its progression. As a result, individual ommatidia are assembled behind the furrow (Lebovitz and Ready 1986; Tomlinson and Ready 1987a, b; Cagan and Ready 1989a, b; Wolff and Ready 1991). Finally, RDN also activates expression of light-sensitive *rhodopsin* genes in the adult retina (Sheng et al. 1997).

While there was initially no clear definition of RDN genes, members in this network were initially grouped together based on two critical criteria. First, loss-of-function mutations in any RDN genes should interfere eye formation and exhibit

severe eye phenotypes that include strongly reduced or complete loss of eyes (Bonini et al. 1993; Cheyette et al. 1994; Mardon et al. 1994; Quiring et al. 1994; Serikaku and O'Tousa 1994; Jang et al. 2003; Dominguez et al. 2004). Second, misexpression of RDN genes in non-retinal tissues should be sufficient to induce ectopic eyes (Halder et al. 1995; Bonini et al. 1997; Chen et al. 1997; Pignoni et al. 1997; Weasner et al. 2007). However, as the field of eye development research has been grown since then, new genes are identified and have been included in this network based on multiple criteria that include genetic, molecular, and biochemical interactions with existing members (Pai et al. 1998; Pan and Rubin 1998; Czerny et al. 1999; Seimiya and Gehring 2000; Curtiss et al. 2007; Braid and Verheyen 2008; Yao et al. 2008; Bessa et al. 2009; Datta et al. 2009). Most members of the RDN are nuclear proteins that control or affect transcription. It includes *eyeless* (*ey*) (Quiring et al. 1994), *twin of eyeless* (*toy*) (Czerny et al. 1999), *eyegone* (*eyg*) (Jun et al. 1998), *twin of eyegone* (*toe*) (Aldaz et al. 2003), *sine oculis* (*so*) (Cheyette et al. 1994; Serikaku and O'Tousa 1994), *optix* (Seimiya and Gehring 2000), *teashirt* (*tsh*) (Pan and Rubin 1998), *tiptop* (*tio*) (Laugier et al. 2005), *distal antenna* (*dan*) (Curtiss et al. 2007), *distal antenna related* (*danr*) (Curtiss et al. 2007), *dachshund* (*dac*) (Mardon et al. 1994), and *homothorax* (*hth*) (Pai et al. 1998). Additionally, two genes *eyes absent* (*eya*) (Bonini et al. 1993) that acts as transcriptional coactivator and belongs to the family of protein tyrosine phosphatase and *nemo* (*nmo*) (Choi and Benzer 1994; Braid and Verheyen 2008) that belongs to protein kinase family are also considered genes of this network (Fig. 1). Recent evidence also indicates that these genes are not only involved in the specification of eye precursors but also controls proliferation and differentiation of retinal precursors as well as specification and/or maintenance of PR neurons (Pignoni et al. 1997; Bessa et al. 2002; Peng et al. 2009; Lopes and Casares 2010). Interestingly, most of these genes have a vertebrate counterpart (Fig. 1), and they are mostly implicated in retinal disorders

In *Drosophila*	In vertebrates	Functional domain(S)
eyeless (ey)	*Pax-6*	paired/homeodomain
twin of eyeless (toy)	*Pax-6*	paired/homeodomain
sine oculis (so)	*Six1/2*	homeodomain
optix	*Six3/6*	homeodomain
eyes absent (eya)	*Eya1-4*	P-S-T/tyrosine phosphatase
dachshund (dac)	*Dach1-2*	winged helix-turn-helix
eyegone (eyg)	*Pax6(5a)*	paired (truncated)/homeodomain
twin of eyegone (toe)	*Pax6(5a)*	paired (truncated)/homeodomain
teashirt (tsh)	*TshZ1-4*	zinc finger
tiptop (tio)	*TshZ1-4*	zinc finger
homothorax (hth)	*Meis1*	TALE homeodomain
distal antenna (dan)	---	pipsqueak
distal antenna related (danr)	---	pipsqueak
nemo (nmo)	*Nlk*	serine/threonine kinase

Fig. 1 Retinal determination network (RDN) genes in *Drosophila* and vertebrates. The table shows a list of the known *Drosophila* RDN genes, its vertebrate homologs, and its corresponding functional domains (Modified from Kumar 2010, 2011)

suggesting that they hold the key to understand normal eye development and its related disorders (Kawakami et al. 2000; Chi and Epstein 2002; Christensen et al. 2008).

Components of the RDN

Eyeless (Ey) and Twin of Eyeless (Toy): The Master Regulators

The first description of *ey* came in 1915 when it was shown that *ey* mutation in *Drosophila* leads to partial or complete loss of compound eyes (Hoge 1915). Cloning and sequencing of the *ey* gene later revealed that it is a homolog to the evolutionary conserved *Pax6* family of transcription factor that contains a paired domain and a homeodomain (Quiring et al. 1994). *Pax6* family of transcription factors in vertebrates is known to play critical roles during eye and central nervous system development (Gehring and Ikeo 1999; Gehring 2004; Kozmik 2005). Pax6/Ey contains two DNA-binding domains: 128-amino acid-long paired domain that are subdivided into helix-turn-helix (HTH) containing amino-terminal PAI and carboxy-terminal RED subdomain and a 60-amino acid-long homeodomain (Ton et al. 1991; Treisman et al. 1991; Walther and Gruss 1991; Jun et al. 1998). Pax6/Ey share 94 percent sequence identity in the paired domain and 90 percent identity in the homeodomain. DNA-binding domain of Pax6 from various species shows high degree of structural similarities and functions despite the fact that different species have differences in the structure and development of the brain and eyes. This is evidenced by formation of ectopic eyes by mouse *Pax6* in *Drosophila* (Halder et al. 1995), which suggests that regulatory mechanisms of this gene have been conserved among different species. Both mouse and human *Pax6* are required for eye formation, which are evidenced by the lack of eye in *Small eye* mice carrying homozygous *Pax6* mutations (Hill et al. 1991). Heterozygous mutations of the human *PAX6* gene are known to cause congenital eye abnormalities known as aniridia and Peters' anomaly and show importance of this transcription factor in eye development (Hanson and Van Heyningen 1995). Both mouse and *Drosophila* show similar *Pax6* expression pattern during development. In mice, *Pax6* is expressed in the spinal cord, in some distinct region in the brain, and in the developing eye. During eye morphogenesis, *Pax6* is first detected at embryonic day 8 in the anterior prospective forebrain. Subsequently, during eye development, it is expressed in the eye vesicle, in the lens, in the differentiating retina, and finally in the cornea (Walther and Gruss 1991). Comparatively, in *Drosophila, ey* expression is first detected in the embryonic ventral nerve cord and some distinct regions of the brain, and later during embryogenesis, it is expressed in the optic lobes and in the primordia of the eye imaginal discs. In subsequent larval stages, *ey* continues to be transcribed in the developing eye imaginal disc. During third and final larval stage, *ey* expression gets largely restricted to the anterior part of the MF in the eye imaginal disc (Fig. 2) where cells are still at the undifferentiated state (Wolff and Ready 1993). Since mutations

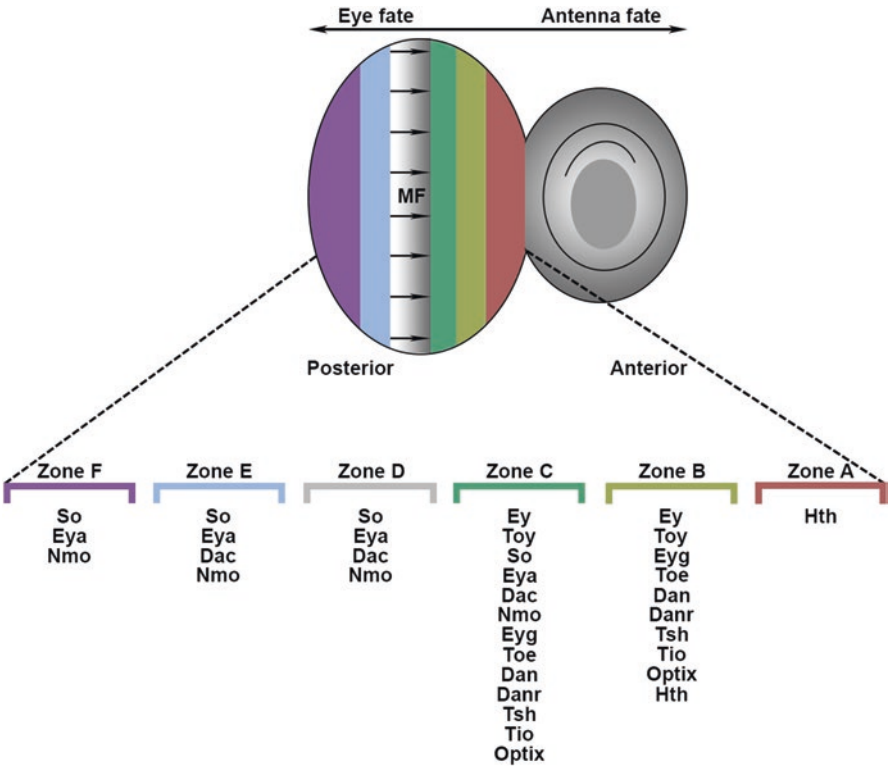

Fig. 2 Expression pattern of RDN genes within the developing eye field of the eye-antennal imaginal disc. Schematic diagram depicting the expression pattern of RDN genes during eye imaginal disc development. Posterior region of the eye-antennal imaginal disc corresponds to the eye fate, whereas anterior region develops into the antenna. The developing eye disc is further divided into six zones (A to F and are represented by different colored horizontal lines) based on the expression pattern of RDN genes, which are listed at the bottom of the figure. The morphogenetic furrow (MF) is colored in gray, and arrows in the MF zone correspond to the movement of MF from posterior to the anterior end of the eye disc (Modified from Kumar 2010, 2011)

in *Pax6/ey* show reduction or complete loss of eyes and because these genes show similarities in DNA sequence as well their expression pattern during eye development, they are highly regarded as master regulator genes involved during eye morphogenesis.

Unlike vertebrates, the fruit fly has an additional *Pax6* homolog called *twin of eyeless* (*toy*). While Ey and Toy share same sequence identity (90%) in the homeodomain of vertebrate Pax6, Ey is more closely related (95%) than Toy (91%) in the paired domain (Quiring et al. 1994; Czerny et al. 1999). However, compared to Ey, Toy shows more sequence similarities to Pax6 proteins outside these domains and are also marked by presence of an additional transactivated carboxy-terminal domain that is absent in Ey but present in Pax6 in vertebrates. Toy locus was mapped

to position 102E1 on the fourth chromosome that is located at the close proximity of the *ey* gene (102D) and consists of nine coding exons that span ~17 kb of genomic DNA. Comparison of the exonic-intronic region of *toy* and *ey* with other *Pax6* genes revealed that *toy* and *ey* may have come into existence as a result of gene duplication most likely during late insect evolution (since only holometabolous insects contain two *Pax6*-like genes) (Czerny et al. 1999). After gene duplication event, both *toy* and *ey* must have acted in parallel until Ey gained one amino acid substitution (Asn to Gly) in the paired domain that has drastically changed the binding affinity and mode of action of the Ey protein. The change in protein sequence after an amino acid substitution in the Ey protein causes a loss of autoregulation of *ey* that is present in *toy* and *Pax6* gene of other species (Plaza et al. 1993). During evolution, *toy* must have been under high selection pressure than *ey* since *toy* is more close to the vertebrate *Pax6* gene than *ey*, and it is also essential for the head development in both vertebrates and flies (Czerny et al. 1999).

Whole-mount in situ hybridization experiment shows that the first RDN gene that is expressed in the embryo is *toy* which is transcribed at stage 5 in the presumptive eye-antennal disc precursors at the posterior procephalic region of the embryonic head (Czerny et al. 1999). Conversely, expression of *ey* begins at stage 9 in the embryo during germband extension and is detected in every segment of the developing ventral nerve cord (Quiring et al. 1994; Czerny et al. 1999). During subsequent development, both genes are expressed in the ventral nervous system of the embryo in different subsets of cells. Moreover, *ey* is expressed in few cells than *toy* but in a spatially restricted manner in both brain hemispheres. However in the developing visual anlagen, *ey* and *toy* get expressed in a very similar fashion, if not identical, in the optic lobe and eye primordia of the late embryo. Expression of both *ey* and *toy* was detected in similar domains during larval stages, and during third larval stage, both of them are expressed in the undifferentiated part of the eye disc in a region that lies anterior to the MF (Fig. 2) (Quiring et al. 1994; Czerny et al. 1999). In addition to its expression in the eye field, *ey* and *toy* are also expressed within the peripodial epithelium (PE; flattened squamous layer of the eye-antennal imaginal disc) during normal development (Baker et al. 2018).

Ey and Toy both are capable to initiate eye developmental program, and it is evidenced by *ey* and *toy* loss-of-function mutants that have variable head and eye phenotype. While hypomorphic alleles of *ey* produce eyeless flies, eye-specific null mutants lack complete head that also includes lack of entire eye-antennal disc (Quiring et al. 1994; Halder et al. 1998; Kronhamn et al. 2002). However, in *ey*$\underline{LB}$ mutant where *ey* does get expressed in the eye field but is absent within the PE also recapitulates *ey* loss-of-function mutant phenotype indicating the importance of PE where *ey* regulates eye development through a completely novel mechanism (Baker et al. 2018). *toy* null and hypomorphic mutants are also mostly headless. However, some escapers form head and compound eyes, but ocelli are always missing (Kronhamn et al. 2002; Punzo et al. 2002). However, loss of *toy* expression only in the PE (but not in the eye field) does not appear to affect compound eye development, but rather it also affects loss and/or mispositioning of the three ocelli (Baker et al. 2018). Epistatic relationship between *toy* and *ey* has been determined by

several different ways that show *toy* functions directly upstream of *ey* in the eye developmental pathway. Both Ey and Toy fulfill some nonredundant functions, and they are evidenced by (1) normal expression of *toy* in the developing eye imaginal discs of *ey* loss-of-function mutants and (2) Toy that is unable to compensate Ey function completely in the *ey* mutant flies. Targeted expression of Ey by tissue-specific enhancer lines produces ectopic eyes in the wings, legs, halteres, and antenna (Halder et al. 1995), whereas misexpression of Toy induces ectopic eyes in the wings, legs, and halteres (Czerny et al. 1999; Salzer and Kumar 2010). Interestingly, targeted expression of Toy induces ectopic *ey* transcription, whereas misexpression of Ey does not induce *toy* transcription. Hence, *toy* acts upstream of *ey* in the transcriptional network governing eye development. Toy was shown to partially rescue eye development as well as to induce ectopic eyes in the absence of *ey* (Punzo et al. 2002; Baker et al. 2018). Toy binds directly to the eye-specific enhancer of *ey*, and this binding is essential for onset of eye development in the embryo. Therefore, *ey* is considered as a master regulator which is activated directly by *toy* and *ey* and in turn activates downstream RDN genes to activate eye developmental pathway.

Sine Oculis (so) and Optix

The homeodomain transcription factor *sine oculis* (*so*) belongs to SIX family of evolutionary conserved homeobox transcription factor found in diverse metazoans that include range of species from flatworms to humans. Mutations in *sine oculis* were first identified and characterized in fruit flies (Milani 1941). It was found to be very interesting since loss of function of *so* was not only affecting compound eye formation but also adversely affected the entire visual system (Fischbach and Heisenberg 1981; Fischbach and Technau 1984; Cheyette et al. 1994; Serikaku and O'Tousa 1994). Subsequent molecular efforts have identified two additional SIX family members, *optix* and *DSix4*, in *Drosophila* (Seo et al. 1999; Seimiya and Gehring 2000). *so* and *optix* are categorized as members of RDN since their loss-of-function mutants show severe defects in eye development and their forced expression is sufficient to induce ectopic eyes (Cheyette et al. 1994; Serikaku and O'Tousa 1994; Pignoni et al. 1997; Seimiya and Gehring 2000; Weasner et al. 2007). However, *DSix4* plays no role during eye development but instead critical for somatic cells of the gonad and fat body development (Kirby et al. 2001; Clark et al. 2006). Homologs of *so*, *optix*, and *DSix4* are found in a wide range of species throughout animal kingdom. However, structural and sequence analysis has further created three SIX protein subclasses, and each subclass contains one of the fly genes and their orthologs (Seo et al. 1999). Successful cloning of *so* from the fruit fly was able to identify homologs in fish, chicken, mice, and humans (Oliver et al. 1995; Bovolenta et al. 1996; Kawakami et al. 1996; Loosli et al. 1998; Seo et al. 1998; Toy et al. 1998; Granadino et al. 1999; Leppert et al. 1999; Zuber et al. 1999). The *Drosophila so* gene is most closely related to murine *Six1/2*, whereas *optix* belongs to *Six3/6*, and *DSix4* is the homolog of *Six4/5* (Seo et al. 1999). So is expressed at

multiple stages during embryonic development. *so* transcript is first detected at stage 5 embryo in the optic primordium of head ectoderm (Cheyette et al. 1994). At stage 9 during germband extension, it is expressed in the optic lobe primordia anterior to the cephalic furrow. Subsequently at stage 12, *so* transcript is detected bilaterally at the segmental boundaries, and at stage 16, its expression gets restricted to four bilaterally positioned larval eye precursors (also known as Bolwig's organ) at the anterior part of the head (Serikaku and O'Tousa 1994). In the larvae, its expression starts at the onset of third instar before the initiation of MF. At this stage, *so* is expressed as a gradient increasing from anterior to posterior side of the MF (Cheyette et al. 1994). *so* expression persists throughout the larval third instar where it is not only restricted to the anterior side but also expressed within and posterior side of the MF (Fig. 2). Additionally, *so* is also expressed in the leg discs but not in the wing and haltere discs (Cheyette et al. 1994).

optix transcript is first detected at the anterior end of the stage 5 blastoderm embryo in a similar pattern as *so* but lies more anteriorly. During germband extension at stage 9, *optix* expression is restricted to the anterior end and is not expressed in the optic lobe primordia. At stage 11, *optix* expression is still limited to the anterior side, whereas at stage 14, it covers the supraesophageal ganglion of the embryonic brain but not in the larval eye precursors (Seimiya and Gehring 2000). During larval eye disc development, *optix* expression starts in the eye disc at the second instar, before the formation of MF. It marks the entire eye disc, but subsequently its expression gets restricted anterior to the MF (Fig. 2). The expression pattern of *optix* looks very similar to *ey* and *toy* expression pattern suggesting that *optix* may play an important role in the early eye development. Additionally, *optix* is also expressed in the wing and haltere discs, but it is not expressed in leg discs (Seimiya and Gehring 2000).

So is required for the development of entire visual system including compound eyes, ocelli, optic lobe, and larval eye precursors in the embryo. In eye-specific mutants where so is not expressed in the eye-antennal disc, MF initiation is blocked, and cell proliferation, retinal differentiation, and PR formation are impaired. This leads to extensive cell death, and as a result adult flies develop without eyes (Cheyette et al. 1994; Serikaku and O'Tousa 1994; Pignoni et al. 1997). so^{1} is considered as the strongest nonlethal and eye-specific loss-of-function mutant which in most cases lacks compound eyes and ocelli. Other mutants too have severe effects in the eye development that includes reduction in eye size and displaying rough eyes (Heitzler et al. 1993; Cheyette et al. 1994). so^{D} (*droplet, drl*) is a dominant negative allele of *so* that has similar phenotype as so^{1} mutant except ocelli are not reduced and it is homozygous lethal (Heitzler et al. 1993). Loss of *optix* function *in* $optix^{1}$ null mutant shows small, kidney-shaped eyes, and therefore it is proposed that *optix* is only required for MF progression but not for the initiation during *Drosophila* retinal development (Li et al. 2013).

Initially it was shown that *so* by itself is not able to induce ectopic eyes (Chen et al. 1997; Pignoni et al. 1997) but rather it interacts with an another RDN gene called *eyes absent* (*eya*). Eya acts as transcriptional co-activator, and its interaction with So is required for So-induced activation of downstream target genes (Pignoni

et al. 1997). Additionally, genome-wide search has uncovered an autoregulatory loop where So binds to its own enhancer and activate itself (Pauli et al. 2005). However, a Gal4 screening was recently done where 219 unique Gal4 driver lines were crossed to UAS-*so* responder line and identified 4 cases where ectopic eyes were formed mainly in the antennal part of the eye-antennal imaginal disc. Interestingly, this part of the eye-antennal disc normally lacks *eya* expression suggesting that induction of retinal tissue by *so* is not only *eya* dependent. This result was also sufficient to prove that *so* by itself is sufficient to initiate eye specification cascade (Weasner et al. 2007). Additionally, *eya* transcriptionally gets activated in response to So during ectopic eye formation, and it was assumed that activation of *eya* forms So-Eya complex and promotes eye specification by activating downstream target gene transcription (Weasner et al. 2007). However, it should be noted that So is not always sufficient to activate *eya* transcription nor the co-expression of *so* and *eya* is always sufficient to promote eye development. Therefore, identification of those factors which mediate *eya* activation by *so* and specification of eye fate downstream of So-Eya complex would be critical in the future to understand eye specification cascade in more details.

Forced *optix* expression on its own is sufficient to induce ectopic eye formation, and this process does not require an interaction of *optix* with *eya* (Seimiya and Gehring 2000). Also, *optix* can induce ectopic eyes in the absence of *ey* but is not able to induce retinal fate in *so* and *eya* mutant background. Induction of ectopic eyes by *optix* was restricted to the antennal disc in addition to the formation of extra ocelli (Seimiya and Gehring 2000). Interestingly, 219 unique Gal4 driver lines were also used here to cross with UAS-*optix* (previously those Gal4 lines were crossed with UAS-*so*; see above) and show that *optix* is also sufficient to induce ectopic eyes in the antennal, wing, and haltere discs (Weasner et al. 2007). In *Drosophila*, binding sites for SIX proteins are very similar and it is likely that So and Optix has common target genes. However, rescue experiments have suggested that Optix cannot substitute for So during eye development (Weasner et al. 2007). This could be due to C-terminal region of the SIX domain which is a non-conserved region and is important in conferring functional specificity (Weasner and Kumar 2009). SIX domain is involved in protein-protein interactions, and therefore functional specificity of So and Optix was provided by having distinct binding partners in the SIX domain (Kenyon et al. 2005; Weasner et al. 2007). For example, So can toggle between an activator and repressor by interacting with either Eya or transcriptional corepressor Groucho (Gro) (Kenyon et al. 2005; Salzer and Kumar 2009), whereas Optix is considered as a dedicated repressor that can only interact with Gro (Kenyon et al. 2005). Functional specificity is further governed by SBP (SO binding protein) which interacts strongly with So, and the zinc-finger containing protein OBP (OPTIX binding protein) binds strongly with Optix (Pignoni et al. 1997; Seimiya and Gehring 2000; Silver et al. 2003; Kenyon et al. 2005).

Eyes Absent (Eya)

eya is a core member of RDN gene family, and as the name suggests, some *eya* mutants lead to eyeless or reduced eye phenotype (Sved 1986). Eya belongs to phosphatase subgroup of the haloacid dehalogenase (HAD) family of transcriptional co-activators that contains one member in *Drosophila* (Eya) and four members in vertebrates (Eya1–4) (Bonini et al. 1993; Hanson 2001; Tootle et al. 2003). This gene is particularly interesting, not only because it does not belong to the transcription factor family but also because it contains both tyrosine and threonine phosphatase activities (Rayapureddi et al. 2003; Tootle et al. 2003; Okabe et al. 2009). The *Drosophila* Eya protein contains proline-serine-threonine (PST)-rich transactivation domain, a moderately conserved threonine phosphatase motif (TPM), and tyrosine protein phosphatase domain in addition to highly conserved C-terminal Eya domain (ED) (Bui et al. 2000; Jin and Mardon 2016). The precise function of phosphatase activity of the Eya during retinal development is still not clear. However, it is recently reported that Eya transactivation domain and TPM domain are essential for normal Eya activity and its function and are required for normal eye development, while phosphatase domain only plays a minor role (Jin and Mardon 2016). The *eya* gene in *Drosophila* is highly conserved to humans which is marked by rescue of eye-specific *eya* null mutant phenotype in *Drosophila* upon expression of vertebrate *Eya2* (Bonini et al. 1993). Eya regulates multiple developmental processes throughout metazoans (Tadjuidje and Hegde 2013). In *Drosophila*, expression of *eya* not only is restricted to the eye but is also broadly expressed in the embryo, subset of cells in the adult visual system, brain, and ovary. Comparatively, several *eya* alleles show loss of ocelli and cause female sterility or lethality supporting the argument that *eya* had other functions in addition to eye development (Bonini et al. 1993; Boyle et al. 1997). In the *Drosophila* eye, loss-of-function mutations of *eya* show failure of MF initiation, massive apoptosis of the eye discs, and complete failure of the eye development, whereas ectopic expression of *eya* is sufficient to induce ectopic eyes (Bonini et al. 1997).

Eya encodes a novel nuclear protein which is first detected in the blastoderm embryo of the developing head but is not expressed in the embryonic eye primordia. During gastrulation, the expression of *eya* gets broadened and covers a wider domain of the dorsal head. It is also not expressed in the first instar larval eye discs, and its first detectable expression in the eye disc is observed during larval second instar. It is expressed as a gradient which is high in the posterior and lateral margins of the eye disc and low in the anterior and central region (Bonini et al. 1993). In the third instar larvae after MF initiation, the gradient expression of *eya* still persists in the eye disc (Fig. 2) (Bonini et al. 1993).

The eya^{1} mutant shows loss of adult compound eyes. However, other external structures seem to be normal that includes adult ocelli which develop from the edges of the eye-antennal disc. In the brain, eya^{1} mutant shows absence of first optic ganglion (lamina), significant reduction of second optic ganglion (medulla), and disorganization of the lobula and lobula plates (Bonini et al. 1993). In eya^{1} mutant disc, the development of eye portion of the disc during third larval instar is arrested, and

as a result no MF is formed. Conversely, the antennal region of the eye-antennal disc develops normally, and larval photoreceptor organ also appears normal (Bonini et al. 1993). Additional alleles of *eya* gene were isolated by ethyl methanesulfonate (EMS) or P-element mutagenesis screening, and most of the newly generated alleles are either lethal at the embryonic stage or semilethal/viable when homozygous. Among the available alleles of *eya*, there are few that show only loss of the adult compound eyes (eya^{1}, eya^{2}). Others show loss of compound eyes and ocelli (eya^{4}) as well as loss of eyes, ocelli, and female fertility (eya^{3cs}) (Bonini et al. 1997). Further analysis of *eya* mutants suggest that loss of *eya* activity does not seem to affect retinal progenitor cell division but leads to cell fate switching from the differentiation state to massive cell death. Larvae of *eya* allele that cause complete loss of adult compound eyes contain reduced eye-antennal imaginal discs where *ey* is still expressed suggesting that *eya* does not act upstream of *ey* (Halder et al. 1998).

The *eya* gene has two different splice isoforms that are identical for much of their sequence except amino terminal sequence (Bonini et al. 1993). The *eya* type I can induce retinal fate when ectopically expressed during larval stages, whereas type II which is the sole *eya* transcript expressed in embryos also displays the same potential to induce ectopic eye formation. This indicates that if expressed in sufficient levels, both type I and type II have the capacity to make an eye (Bonini et al. 1997). Induction of ectopic eyes by *eya* overexpression is observed in the antenna, legs, and wings (Bonini et al. 1997), and by using other imaginal disc-specific Gal4 drivers, *eya* can also induce ectopic eyes in the halteres and head (Salzer and Kumar 2010).

Dachshund (Dac)

dac was discovered in a genetic screen that was conducted to find novel regulators that modify PR differentiation of the eye through modulating *Egfr* activity. The gene was named *dachshund* since loss-of-function mutants show severely truncated legs and reduction or absence of eyes (Mardon et al. 1994). Dac encodes a novel nuclear protein which is conserved throughout much of the metazoans and required for normal eye development in *Drosophila*. The vertebrate homologs of *dac* are *Dach1/2* and proto-oncogenes *Ski/Sno* which are designated as transcriptional repressors (Hammond et al. 1998). *Drosophila* Dac protein contains two conserved domains: Dachshund domains 1 and 2 or DD1 and DD2. DD2 in Dac is required to facilitate DD1 function and forms a complex with Eya, although neither of these functions are critical for eye development (Pappu et al. 2005).

dac is expressed at the posterior margin of the third instar eye disc prior to MF initiation and neural development. It is strongly expressed immediately anterior and posterior to the furrow throughout MF progression (Fig. 2). Posterior to the furrow, it is expressed in the PR cells R1, R6, and R7 as well as the cone cells. It is expressed primarily to the region of eye disc which gives rise to the retina and not in the periphery of the disc which forms head cuticles. Apart from its expression in the eye disc, *dac* expression is also seen in leg discs during early stages of leg disc

development. Additionally, *dac* is also expressed in the antennal and wing imaginal discs. *dac* expression is also detected in the embryonic central nervous system and in the optic lobe of the larval brain.

Several *dac* alleles have reduced viability. For example, a week dac^{P} allele that is homozygous viable shows reduced and rough eyes, whereas null mutants of *dac* (dac^{4}, dac^{1}, dac^{3}) show either severely reduced eyes or eyes are absent (Mardon et al. 1994). In the absence of *dac* activity, MF remains at the posterior margin of the eye-antennal disc and shows MF progression is not affected in *dac* mutants (Mardon et al. 1994). Size of the eye disc in *dac* mutants is normal suggesting that cellular proliferation is not affected. This is quite interesting since loss of *ey* and other reduced-eye mutants, size of eye discs are significantly reduced (Bonini et al. 1993; Heberlein et al. 1993). *dac* is also not involved in PR differentiation but required cell autonomously for some aspects of ommatidial assembly. Additionally, *dac* mutants show defects in genital disc formation, mushroom body, and antennal development (Kurusu et al. 2000; Martini et al. 2000; Noveen et al. 2000; Dong et al. 2001, 2002).

Misexpression of *dac* in non-retinal tissues (antennal and leg imaginal discs) induces ectopic eye formation (Shen and Mardon 1997). Additionally, *dac* acts downstream of *ey* during retinal specification (Shen and Mardon 1997) since *dac* is not required for *ey* expression, but misexpression of *ey* induces *dac* expression. *dac* induces ectopic retinal development by targeted *ey* expression suggesting that *dac* and *ey* are intimately related and they function together to control eye specification events (Chen et al. 1997; Shen and Mardon 1997). Dac directly interacts with Eya, and they activate each other's transcription, and synergistic misexpression of *dac* and *eya* strongly induces ectopic retinal development (Chen et al. 1997).

Teashirt (Tsh) and Tiptop (Tio)

The *Drosophila* RDN gene *teashirt* (*tsh*) and its paralog *tiptop* (*tio*) are nuclear proteins that encode zinc-finger transcription factors (Laugier et al. 2005). They play important roles during eye development which includes promoting cell proliferation in the anterior region of the eye field as well as inducing ectopic retinal fate in non-retinal precursors (Pan and Rubin 1998; Bessa and Casares 2005; Datta et al. 2009). This gene pair is found in all *Drosophila* species but presents only as a single gene in other insects indicating that gene duplication event occurred during the evolution of Drosophilidae (Laugier et al. 2005; Bessa et al. 2009). *tsh* was originally discovered as a homeotic gene which is necessary for specifying the trunk segments in the embryo. Therefore, loss-of-function mutations in *tsh* showed trunk to head transformation, whereas forced expression showed head to trunk transformation (Fasano et al. 1991; de Zulueta et al. 1994). Later, *tsh* was also involved in patterning other tissues, such as the salivary gland and midgut in the embryo (Mathies et al. 1994; Henderson et al. 1999). Additionally, *tsh* is involved in specification and patterning of adult appendages such as the leg, wing, and eye (Erkner et al. 1999, 2002; Wu and Cohen 2000, 2002; Soanes et al. 2001; Bessa et al. 2002,

2009; Singh et al. 2002, 2004; Bessa and Casares 2005). Vertebrate *teashirt* family genes (*Tshz*) were also identified, and mouse *Tshz* was sufficient to rescue trunk phenotype in *Drosophila* suggesting that this gene is conserved.

tsh expression is first detected at stage 6 embryos (Alexandre et al. 1996), whereas *tio* expression is first detected at stage 10 embryos (Laugier et al. 2005). At stage 10, *tsh* is expressed in the trunk region, whereas *tio* is expressed in the posterior part of the embryo specifically to the Malpighian tubule primordia and a subregion of the hindgut primordia (Laugier et al. 2005). However, co-expression of these two proteins was seen at stage 12 in some cells of the CNS and epidermis. Also, during development co-expression was increased, but they still maintain their distinct expression pattern (Laugier et al. 2005). Interestingly, *tsh/tio* is not expressed in the embryonic eye-antennal disc primordia (Bessa and Casares 2005). In the larval eye-antennal imaginal disc, *tio* and *tsh* expression completely overlaps and is expressed in an identical pattern in the retina (Laugier et al. 2005; Bessa et al. 2009; Datta et al. 2009). *tsh* is detected as early as larval first instar in the entire disc proper overlapping with *hth* and pro-eye gene *ey*. In the larval second instar, *tsh* expression is retracted toward the anterior three quarter of the disc, and at the third instar, *tsh* expression covers two-thirds of the disc anterior to the MF and is repressed posterior to it (Pan and Rubin 1998; Singh et al. 2002). In early third instar, *tsh* is co-expressed with *hth* in a three- to four-cell-wide stripe in the eye disc suggesting that during early phases of eye disc development, *tsh* induces *hth* expression (Fig. 2). *tsh* is also expressed in the wing disc at late larval third instar where expression is seen as a proximal ring around wing pouch and in most of the notum largely overlapping with *hth* expression (Pai et al. 1998; Azpiazu and Morata 2000; Casares and Mann 2000). *tsh* is also expressed in the antennal disc of the anteroproximal region (Bhojwani et al. 1997; Pan and Rubin 1998), and it overlaps with *hth* which is expressed in the proximal region (Rieckhof et al. 1997; Casares and Mann 1998; Pai et al. 1998).

Since *tio* and *tsh* both are redundant to each other, null mutants of only *tio* are homozygous viable, and loss of function of both genes shows no obvious effect in the structure of the eye (Pan and Rubin 1998; Singh et al. 2002; Laugier et al. 2005). However, a report suggests that knockdown of *tsh* functions by expressing *tshRNAi* in the eye disc upon combination of *ey*-Gal4 and *arm*-Gal4 shows reduced eye phenotype (Bessa and Casares 2005). In *tio* mutants, *tsh* is ectopically expressed in a region where *tio* is normally expressed, and in *tsh* mutants ectopic *tio* expression is seen in the trunk (a region where *tsh* is normally expressed) suggesting that these genes mutually repress each other's expression. Further, ectopic expression of *tio* in the trunk represses *tsh* and vice versa.

Ectopic expression of either Tsh or Tio gives a similar phenotype suggesting that both act on similar targets (Laugier et al. 2005). Targeted expression of *tsh* is sufficient to induce ectopic eyes in the antennal disc. *tsh* induces expression of key RDN genes such as *ey*, *so*, and *dac*, and ectopic retina development by *tsh* depends on the activity of *eya* and *so* (Pan and Rubin 1998). Since early eye primordium is further subdivided into dorsal and ventral parts (Cho and Choi 1998; Dominguez and de Celis 1998; Papayannopoulos et al. 1998; Cavodeassi et al. 1999), many genes show

dorsoventral asymmetry in their either expression or function during eye development. Although *tsh* is expressed symmetrically in both dorsal and ventral compartments of the eye disc, its function shows dorsoventral asymmetry: in the ventral region, *tsh* suppresses eye development, whereas in the dorsal region of the eye disc, it promotes eye development (Singh et al. 2002). The phenotype observed here by overexpression of *tsh* might be dose-dependent since additional copies enhance the phenotype (Singh et al. 2002).

Eyegone (Eyg) and Twin of Eyegone (Toe)

Alternate splicing of Pax6 in vertebrates leads to the formation of Pax6(5a) which is marked by presence of only RED (and no PAI) domain in addition to Prd-class homeodomain (HD) (Jun et al. 1998). Pax6(5a) binds to the DNA either through its RED or HD and has different DNA-binding specificities than canonical Pax6 (Jun et al. 1998). In *Drosophila*, Pax6(5a) is also present, but unlike vertebrates, it does not form as a result of alternate splicing of Pax6 but rather encodes two separate genes: *eyegone* (*eyg*) and *twin of eyegone* (*toe*). In vertebrates, Pax6 and Pax6(5a) play different roles during eye development which is also true in case of *Drosophila.* For example*, ey* and *toy* (*pax6* homolog) are involved primarily in *Drosophila* eye specification, whereas eyg promotes cell proliferation (Chao et al. 2004; Dominguez et al. 2004). Ey mainly acts as a transcriptional activator, whereas Eyg acts as a dedicated repressor (Punzo et al. 2001, 2004; Yao and Sun 2005).

Spatial and temporal expression pattern of *eyg* is unique unlike other *Pax* genes in *Drosophila. eyg* expression is expressed in the embryo as well as leg, wing, and eye-antennal discs in the larvae (Jones et al. 1998; Jun et al. 1998; Aldaz et al. 2003). *eyg* and *toe* transcripts are first observed at stage 9 in the embryo in salivary gland precursors and small group of cells within the dorsal head. By the embryonic stage 10, both transcripts show identical expression pattern within the posterior and anterior thoracic and abdominal segments (Jones et al. 1998; Jun et al. 1998; Yao et al. 2008). At stage 12 in the embryo, *eyg* and *toe* transcripts are extended to the antennal organ (AO) as well as to the leg disc primordia. However, at late embryonic stage, both *eyg* and *toe* transcripts are observed in the eye-antennal imaginal disc primordia in same cells that expresses *ey* and *toy* (Quiring et al. 1994; Jones et al. 1998; Jun et al. 1998; Czerny et al. 1999; Yao et al. 2008). In the developing larval eye-antennal disc, both *eyg* and *toe* transcripts show similar expression patterns. In the antennal part of the disc, both transcripts are localized to the medial and distal segments, whereas in the eye part, they are expressed anterior to the MF (Fig. 2) (Dominguez et al. 2004; Yao et al. 2008). Interestingly, unlike similar expression pattern observed in embryos, the expression pattern of *eyg* and *toe* is different from *ey* and *toy* expression in the eye-antennal disc. *ey* and *toy* are broadly expressed in the eye disc, whereas *eyg* and *toe* are restricted to the dorsoventral compartment boundary and do not extend laterally (Quiring et al. 1994; Czerny et al. 1999; Dominguez et al. 2004; Yao et al. 2008). This difference in expression pattern is likely associated with the requirements of *eyg* (and probably *toe*) for

Notch-mediated control of cell proliferation versus tissue specification by *ey* and *toy*. Apart from their expression in the eye-antennal disc, both *eyg* and *toe* transcripts are also found in an identical pattern in the leg primordium as well as cells of the salivary gland (Jones et al. 1998; Yao et al. 2008).

Weak loss-of-function mutants of *eyg* show reduction or absence of adult eyes, whereas in strong loss-of-function mutants of *eyg*, adults do not hatch from their pupal case, and although they appear normal, their heads are severely reduced in size (Jang et al. 2003). The null mutant allele $eyg^{M3\text{-}12}$ shows headless phenotype where all eye-antennal disc derivatives are missing (Jang et al. 2003) and the phenotype resembles *ey* or *toy* mutants (Jiao et al. 2001; Kammermeier et al. 2001; Kronhamn et al. 2002). In contrast to *eyg*, *toe* loss-of-function mutants are unavailable making it difficult to define its role during retinal development. However, reports suggest that *toe* miRNA can fully substitute *toe* loss-of-function mutants (Yao et al. 2008). When *toe* miRNA is expressed in the eye disc by using *eyg-GAL4*, no obvious phenotype in the eye was observed suggesting that although Toe levels are eliminated in this condition, endogenous level of Eyg is sufficient to fully support the eye development (Yao et al. 2008). This is further supported by an experiment where both Eyg and Toe levels are simultaneously compromised and that results in the blockage of both compound eye and head development (Yao et al. 2008).

Forced expression of *eyg* or *toe* is sufficient to induce ectopic eye formation (Jang et al. 2003; Yao et al. 2008). However, *toe*- or *eyg*-induced ectopic eyes are always located in the ventral part of the endogenous eyes. The formation of an additional eye field is also detected during forced expression of *eyg* or *toe* in the larval eye disc (Jang et al. 2003; Yao et al. 2008). Additionally, ectopic MFs are also detected usually at the dorsal and ventral sides between eye and antennal discs as well as dorsal and ventral poles of the eye disc (Jang et al. 2003). However, percentage of extra PRs are higher than extra eyes in adults suggesting that when endogenous eye and ectopic eye fields grow, they often fuse together. Forced expression of *eyg* but not *toe* is sufficient to rescue eyg^{1} mutant phenotype in the retina suggesting that they are functionally diverged after the gene duplication event (Yao et al. 2008).

Although forced expressions of both *eyg* and *ey* induce ectopic eyes, they are transcriptionally independent, and neither *eyg* nor *ey* expression is strongly dependent on each other. This shows that compared to other RDN genes, *eyg* works independently with *ey* to induce ectopic eye formation. However, their co-expression significantly enhances the phenotype (Jang et al. 2003). Nevertheless, higher level of *ey* and *eyg* than its normal endogenous level can functionally substitute for each other by partially rescuing each other's loss-of-function mutant phenotype (Jang et al. 2003).

Homothorax (Hth)

homothorax (hth) is a homolog of murine proto-oncogene *Meis1* in *Drosophila* that encodes a homeodomain of three-amino-acid-loop-extension (TALE) subfamily of transcription factor (Moskow et al. 1995; Rieckhof et al. 1997). Hth has a nuclear

localization signal and contains two conserved domains: N-terminal conserved MH (Meis and Hth) domain and C-terminal homeodomain (HD) (Rieckhof et al. 1997; Pai et al. 1998; Ryoo et al. 1999; Jaw et al. 2000; Noro et al. 2006). Hth is known to interact directly with Extradenticle (Exd) via its MH domain, and this interaction is required by Hth for its nuclear localization to regulate transcription of downstream target genes (Abu-Shaar et al. 1999; Stevens and Mann 2007).

Hth is ubiquitously and weakly expressed in all cells of the eye-antennal imaginal disc at second instar larval stage. However, during third instar larval stage, Hth is expressed strongly in the anterior region surrounding the eye field including ptilinum, ocellus, and head capsules. Hth is weekly expressed in the posterior and lateral margins of the eye (Pai et al. 1998; Pichaud and Casares 2000). Very weak Hth expression is also detected in the posterior region composed of differentiated PRs (Fig. 2). In the antennal disc, it is expressed in all but the arista region. In addition to the eye-antennal disc, *hth* is also expressed in the notum, wing hinge, and ventral pleura of the wing disc and peripheral region of the leg discs.

hth loss-of-function alleles are mostly embryonic lethal, and therefore to examine its role in the retinal development, *hth* mutant clones were generated by FLPase-based mitotic recombination during larval stages. Clones generated only in the ventral head capsule resulted in ectopic eye formation suggesting that Hth acts to suppress eye formation. Clones induced in the eye-antennal discs of the third instar larvae show ectopic PR differentiation and local outgrowth (Pai et al. 1998; Pichaud and Casares 2000). However, ectopic PR differentiation was only found at the ventral margin, whereas it is absent at the dorsal margin of the eye-antennal disc (Pai et al. 1998).

Ectopic expression of *hth* by using *dpp-gal4* (expressed at the posterior margin of the eye disc where MF initiates) completely suppressed eye development (Pai et al. 1998; Pichaud and Casares 2000). Interestingly, Hth suppresses eye development by blocking MF progression and possibly also MF initiation (Pai et al. 1998; Pichaud and Casares 2000).

Nemo (Nmo)

Drosophila nemo (*nmo*) was initially identified as a gene required for ommatidial rotation during eye development (Choi and Benzer 1994). Nmo is the founding member of Nemo-like kinase that belongs to the family of proline-directed serine-threonine kinases (Choi and Benzer 1994). Nemo-like kinases are highly conserved from flies to mammals and display various developmental roles throughout metazoans that include endoderm induction in *C. elegans* (Meneghini et al. 1999), anteroposterior patterning and neurogenesis in zebrafish (Thorpe and Moon 2004; Ishitani et al. 2010), and hematopoiesis in mice (Kortenjann et al. 2001).

nmo shows a dynamic expression pattern throughout the eye-antennal imaginal disc development in the larvae (Choi and Benzer 1994; Braid and Verheyen 2008). At second instar larval stage, *nmo* is ubiquitously expressed in the peripodial cells of the eye disc, whereas at the mid- and late second instar, *nmo* expression coincides

with *ey* and *eya* in the posterior part of the eye disc. As the larvae grow and reach third instar, co-expression of *nmo* with *eya* gets extended to the anterior edge of the MF (Fig. 2). In the third instar eye disc, *nmo* is expressed in the ocellar primordia at the anterior-dorsal region together with *eya*. Notably, *hth* expression is absent at this region, whereas *hth* is expressed at the posterior margin where *nmo* is repressed (Braid and Verheyen 2008). In the antennal disc, *nmo* is expressed in the aristal and Johnston's organ precursors, and here it is co-expressed with the proneural gene *ato*.

nmo mutants show distinct compound eye phenotypes that include small, long, and narrow eyes in addition to the defect in ommatidial patterning (Choi and Benzer 1994). *nmo* synergistically interacts with *ey*, *eya*, *so*, and *dac* to promote normal retina development and enhances their ability to transform head, wing, and leg tissues into ectopic eyes (Braid and Verheyen 2008). High levels of Nmo alone can induce anterior head-to-eye transformation by inducing *dac* and *eya* expression (Braid and Verheyen 2008). Nmo does not molecularly associate with these RDN genes by not affecting their transcription levels but rather interacts with them at the protein levels and acts as a positive mediator of the RDN gene activity during eye-antennal imaginal disc development (Braid and Verheyen 2008; Morillo et al. 2012).

Distal Antenna (Dan) and Distal Antenna Related (Danr)

Fernández/distal antenna (Dan) and Hernández/distal antenna related (Danr*)* belong to pipsqueak (psq) motif (a DNA-binding motif) containing transcription factors present in fungi, sea urchins, nematodes, insects, and vertebrates. They are associated with multiple roles during development that include chromatin structure regulation and cellular memory (Couderc et al. 2002; Siegmund and Lehmann 2002; Lehmann 2004). Both *dan* and *danr* are capable of inducing antennal fate in the distal leg structures and single and/or double loss-of-function mutants of *dan* and *danr* transform distal antenna into a leg (Emerald et al. 2003; Suzanne et al. 2003). Both *dan* and *danr* are also involved during eye specification process, and like most of the RDN genes, forced expression of *dan* or *danr* in the antennal region of the eye-antennal disc induces ectopic eye formation (Curtiss et al. 2007). Both Dan and Danr physically interact with Ey and Dac to regulate the activity of proneural gene *atonal (ato)* and *Egfr* signaling in the differentiated PRs of the eye (Curtiss et al. 2007).

dan and *danr* transcript is first observed at the cellular blastoderm stage of the embryo in a large central domain. During germband elongation at stage 8, *dan* becomes strongly expressed in the presumptive neuroectoderm at the ventral region. At embryonic stage 10, *dan* expression is seen in the neuroblasts as well as in the neuroectoderm and in the ventral nerve cord region. Its expression is maintained in the ventral cord and brain till the end of embryogenesis. *danr* is expressed in a very similar pattern to that of *dan* in the embryo, particularly in the neuroectoderm and in the ventral nerve cord till the end of embryogenesis. In the larvae, both *dan* and *danr* are expressed in the developing eye-antennal disc. In the eye disc, *dan* expression is first detected in the early larval third instar in cells surrounding the MF. As MF migrates from posterior to the anterior end, both *dan* and *danr* are expressed at

high levels in the anterior end of the MF and at low levels in the differentiating PRs at the posterior end (Fig. 2) (Suzanne et al. 2003; Suzanne 2004; Curtiss et al. 2007). Within differentiated PRs at the posterior end of MF, *dan* is expressed at a higher level than *danr*. *dan* and *danr* expression pattern resembles to the *eya*, *so*, and *dac*, and it considerably overlaps with *ey* expression pattern (Curtiss et al. 2007). Apart from the eye disc, *dan* and *danr* are also expressed in the larval third instar antennal disc as well as in the wing and labium at the end of pupal stage (Suzanne 2004).

To determine the role of *dan* and *danr* during eye development, the following mutants were used: $danr^{ex35}$ mutant which is a null mutant of *danr*, *dan* $danr^{ex56}$ mutant which is a double null mutant of *dan and danr*, and dan^{ems3} mutant which is a loss-of-function *dan* allele (Curtiss et al. 2007). Around 90 percent of these mutants die during larval and pupal stages, and 10 percent escapers survive only for a few hours and show antenna-to-leg transformation (Emerald et al. 2003; Suzanne et al. 2003). Loss of *danr* shows smaller and rough eyes, whereas loss of both *dan* and *danr* results in rough eyes suggesting that both *dan* and *danr* are involved in eye development. Additionally, $danr^{ex35}$ and *dan* $danr^{ex56}$ mutants show defects in ommatidial patterning as well as in the recruitment and identity of differentiated PRs in the eye. Interestingly, Dan is expressed at higher levels in $danr^{ex35}$ clones in the eye disc, whereas low expression of Danr is observed in the dan^{ems3} clones generated at the anterior end to the MF (Curtiss et al. 2007). This suggests that both Dan and Danr regulate each other's expression: Danr represses Dan expression, whereas Dan promotes Danr expression anterior to the MF.

Forced expression of both *dan* and *danr* by using *Distal-less (Dll)-Gal4* (used to drive expression in the distal region of the antennal disc) is sufficient to induce ectopic eyes in the antenna (Suzanne et al. 2003; Curtiss et al. 2007). Ectopic eyes generated by both *dan* and *danr* misexpression show normal retinal morphology, containing several ommatidia where PR differentiation occurred normally (Curtiss et al. 2007). Additionally, misexpression of both *dan* and *danr* is sufficient to induce and maintain *ey* expression in the antennal region of the eye-antennal disc (Curtiss et al. 2007).

Specification of Eye Fate by Genetic Interactions Between RDN Genes

Extensive studies in the field of eye development in *Drosophila* have revealed novel insights into the transcriptional regulation of RDN which is valuable for studying early steps of tissue specification. RDN is characterized by hierarchical cascade of transcriptional activation and repression in addition to having autoregulatory and feedback loops. An interesting fact about each member of RDN is that a single gene can be involved in multiple steps and controls the expression of multiple genes within this network. In this part, we will review genetic interactions among different RDN genes within the network as well as outside the network for specifying eye fate.

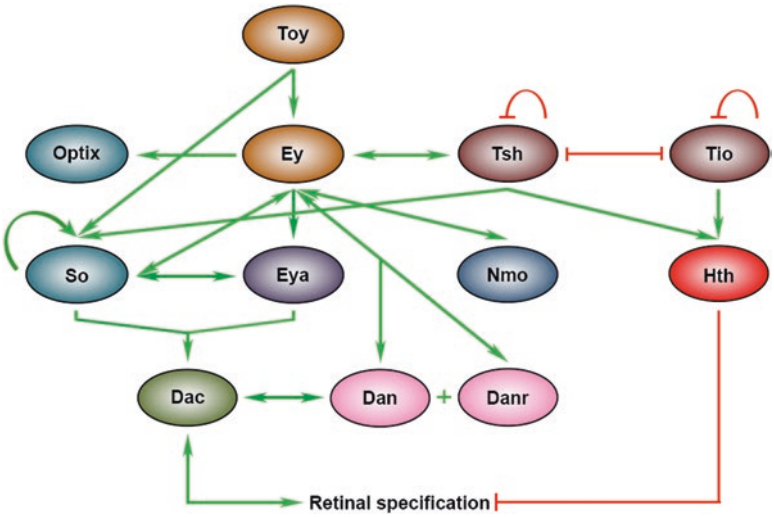

Fig. 3 The working model of retinal determination network. Schematic representation of all known RDN genes and their interactions that lead to activation of retinal specification during eye imaginal disc development. Green arrows represent transcriptional activation, whereas red lines show repression (Modified from Kumar 2009a)

Initiation of the Eye Fate by Multiple Interactions of *ey* and *toy*

Initiation of an eye fate occurs after the activation of *ey* gene in the embryo. However, expression of *toy* starts earlier than *ey* in the brain region of the developing embryo, and through genetic and biochemical experiments, it was shown that *toy* acts upstream of *ey* (Fig. 3) (Czerny et al. 1999). *toy* is the first zygotically expressed gene involved during eye development in *Drosophila*. *toy* is activated by combined action of maternal patterning genes and zygotically active gap genes in the embryo. Toy initiates *ey* expression by binding directly to the eye-specific enhancer of the *ey* gene and controls eye developmental program in the embryo (Fig. 3). Misexpression of *toy* produces ectopic eyes and induces *ey* transcription (Punzo et al. 2002; Baker et al. 2018), whereas targeted expression of *ey* was not sufficient to induce *toy* transcription in the ectopic eyes (Czerny et al. 1999). However, although *toy* acts upstream of *ey*, it depends on Ey function to induce retinal fate since *ey* loss-of-function mutants show inability of *toy* to induce ectopic eyes on legs and wings. One reason could be that Toy expression might be sufficient to promote transcription of downstream targets genes but it is unable to promote at that level which are needed to maintain eye development in *ey* loss-of-function mutant retinas (Baker et al. 2018).

ey acts upstream of *so* and *eya* during retinal development since so^{1} and eya^{1} mutants exhibit normal Ey expression, whereas in ey^{2} mutant, expression of So and Eya was not detected (Fig. 3). Additionally, So and Eya expression is induced in the

ectopic eyes generated in the wing disc proper (where So and Eya are normally not expressed) by *ey* misexpression (Halder et al. 1998). However, Ey requires function of both *so* and *eya* during ectopic eye formation since misexpression of *ey* was unable to induce ectopic eyes in so^{1} and eya^{1} mutant background. Interestingly, since *ey* controls the expression of *so* and *eya*, they both appear to be independent targets of Ey (Fig. 3). In comparison to the *ey*, early expression pattern of *toy* was similar to the *so* expression in the embryonic head. However, *so* is expressed normally in the embryonic head in *toy* mutants, and *toy* was also found to be expressed normally in the null allele of *so* suggesting that both *so* and *toy* do not regulate each other during embryonic head development in *Drosophila* (Halder et al. 1998). However, in the case of eye-antennal imaginal disc in the larvae, only *toy* (but not *ey*) is expressed in the ocellar region, and ey^{2} mutants hamper compound eye development but not the ocellar development. This indicates that *so* and *eya* which are expressed in the ocellar precursors of the eye discs are not controlled by *ey.* Genomic analysis by using eye-specific *so10* enhancer (whose sequence is deleted in so^{1} mutant) shows that both Ey and Toy bind to this enhancer through their paired domain at different sites (Niimi et al. 1999; Punzo et al. 2002). Toy binding sites are required for ocelli development, whereas Ey binding sites are required for the development of compound eyes (Punzo et al. 2002).

Expression of Ey in the PE regulates *dpp* expression and triggers morphogenetic furrow (MF) initiation, and therefore in *eyLB* mutants (where *ey* is only expressed in the eye field but not in the PE), *dpp* expression is lost along the posterior margin of the MF, and it recapitulates *ey* mutant phenotype (Baker et al. 2018). Restoration of *dpp* expression to the PE in *eyLB* mutant eye disc is sufficient to rescue eye mutant phenotype back to its normal state (Baker et al. 2018). Loss of *toy* expression just within the PE does not appear to affect compound eye development, but rather it affects loss and/or mispositioning of the three ocelli (Baker et al. 2018). Furthermore, developmental time during which Ey controls *dpp* expression within the PE has also been calculated, and it was found that the critical period is between the middle of the first larval instar to the middle of the second larval instar stage. Therefore, if Ey expression in the PE is removed before or after this developmental time window, no recapitulation of the eye phenotype will be seen (Baker et al. 2018).

eya activity is required for *ey*-dependent ectopic eye formation, and therefore *eya* expression was observed ectopically in those regions where *ey* induced ectopic eyes such as antennal region of the eye-antennal disc as well as the leg and wing discs (Bonini et al. 1997). It is important to note that Eya is normally not expressed in cells of the antennal, leg, and wing discs. Additionally, no ectopic eyes were observed in antennal, leg, and wing discs of animals that have misexpression of *ey* in the *eya* null mutant background suggesting that *eya* gene activity is essential for *ey*-mediated ectopic eye formation. Since, *eya* can induce ectopic eye formation by itself, it requires *ey* gene activity to direct eye development in both the head region and legs (Bonini et al. 1997). Interestingly, although Ey binding sites are observed in the regulatory region of *eya* gene, Ey binding regions of *eya* do not regulate *eya* expression in a reporter assay. Therefore, it cannot be excluded that other regulatory regions of *eya* are required for eye-specific expression of *eya* (Ostrin et al. 2006).

optix on the other hand does not require *ey* to induce retinal fate, and therefore no ectopic *ey* expression is seen during ectopic eye formation by *optix*, and *optix* is also able to induce ectopic eyes in an ey^2 mutant background (Seimiya and Gehring 2000). Genome-wide studies indicate that Ey is a direct target of Optix and misexpression of *ey* is sufficient to induce *optix* expression (Ostrin et al. 2006). Therefore it is still not clear how *ey* and *optix* are associated during normal eye development.

Ectopic eyes formed by targeted expression of *dac* look remarkably similar to that of *ey* misexpression. However, misexpression of *ey* in the antennal, leg, and wing discs is sufficient to induce ectopic *dac* expression suggesting that *ey* positively regulates *dac* transcription. Additionally, *ey* is unable to induce ectopic eye formation in the *dac* mutant background suggesting that *dac* activity is essential for *ey*-mediated ectopic eye formation. *dac* works downstream of *ey* during eye development and is not essential for *ey* expression since *ey* is normally expressed in *dac* null mutant background (Shen and Mardon 1997). Interestingly, ectopic *dac* expression in the antennal disc is also sufficient to induce *ey* expression suggesting that *dac* also works upstream of *ey* during retinal development (Shen and Mardon 1997).

Since ectopic eye formation by *tsh* overexpression looks similar to that of *ey* misexpression, a relationship between *ey* and *tsh* was observed and found that *tsh* is sufficient to induce ectopic *ey* expression in the antennal region of the eye-antennal disc where ectopic retinal determination occurs. Additionally *tsh* expression is also induced during *ey* misexpression suggesting that *tsh* works both upstream and downstream of *ey* during retinal development (Singh et al. 2002).

Loss-of-function mutant of *nmo* rescues multiple aspects of *ey* mutant phenotype indicating that *nmo* is involved in Ey-mediated eye development process. Ectopic eyes induced by *ey* misexpression in the head, antennae, legs, and wings show respecified retinal cells having ectopic *nmo* expression. Interaction between *ey* and *nmo* was further investigated upon ectopic *ey* expression in *nmo* mutant background which shows significant reduction in the formation of ectopic eyes indicating that *nmo* acts as a positive component of *ey*-mediated retinal determination. Furthermore, misexpression of *nmo* alone can respecify head precursors as eye cells, whereas targeted co-expression of *ey* and *nmo* significantly increased the frequency of ectopic eye formation (Braid and Verheyen 2008).

So-Eya Complex Further Dictates the Eye Development

So and Eya functionally acts together to form a transcriptionally active complex to control multiple steps during retinal development. For example, in the eye-antennal disc, loss of this complex leads to overgrowth phenotype in the undifferentiated epithelium of the eye disc indicating that it regulates proliferation and contributes in regulating the size of the eye disc. Both *so* and *eya* may directly bind and regulate genes required for MF initiation and its progression. They also control *dac* and *dpp* expression in the third instar eye disc. In the posterior end to the MF, both *so* and *eya* are also required for neuronal development (Serikaku and O'Tousa 1994; Pignoni et al. 1997; Niimi et al. 1999). Misexpression of both *so* and *eya* is

sufficient to induce ectopic eyes at a relatively low frequency, whereas their forced co-expression leads to a strong synergistic increase in the formation and size of ectopic eyes particularly in the antennal region of the eye-antennal disc. This provides a strong genetic evidence that both *so* and *eya* functionally act together. The direct interaction between So and Eya was confirmed in a yeast two-hybrid system and also in vitro by using ^{35}S-methionine-labeled transcription/translation assay (Pignoni et al. 1997). Therefore, based on above observation, a model of So-Eya complex during eye development was proposed. According to this model, So binds to the DNA through its homeodomain and Eya that does not have DNA binding property of its own, binds to So, and acts as transcriptional co-activator for downstream target genes (Pignoni et al. 1997). Since Eya belongs to the family of protein-tyrosine phosphatase (Tootle et al. 2003), it may be possible that Eya regulates So phosphorylation by binding to it and So phosphorylation may mechanistically be important during eye specification process.

Induction of ectopic eyes does not require co-expression of *so* and *eya,* but targeted *so* expression leads to the activation of *eya* transcription suggesting that after *so* gets activated by *ey* and *toy*, it activates *eya* expression (Fig. 3). So-Eya complex promotes eye specification by activating target gene transcription (Fig. 3). However, it should be noted that *so* does not always induce *eya* transcription nor the co-expression of *so* and *eya* always sufficient to induce retinal fate during eye development (Weasner et al. 2007). Therefore, it is possible that there are additional factors involved which activate *eya* by *so*, and these factors are critical to mediate retinal determination by So-Eya complex.

So may function both as transcriptional activator and repressor during eye development based on expression level of cofactors it binds. Groucho (Gro) is a potent transcriptional repressor that has a binding affinity with So, and it is proposed that when Eya levels are not high, Gro interacts with So and promotes downregulation of target genes involved during retinal development. The Eya and Gro binding sites are however not the same, and So-Gro complex is inhibited when Eya levels are high (Silver et al. 2003).

Synergistic Action of Eya and Dac Promotes Eye Formation

Since *dac* and *eya* both are sufficient to induce ectopic eye formation, *eya* expression in the eye disc is independent of *dac* function, whereas *dac* expression requires *eya* activity suggesting that *dac* may act downstream of *eya* during eye development (Fig. 3) (Chen et al. 1997). Misexpression of *dac* or *eya* alone is sufficient to induce ectopic eyes at a relatively low frequency, whereas co-expression of *dac* and *eya* synergistically increased ectopic eye formation in the head, legs, wings, and dorsal thorax (Chen et al. 1997). Co-expression of *eya* and *dac* induced ectopic eyes even in those regions (e.g., in dorsal thorax) where misexpression of *dac* and *eya* alone is not sufficient to induce retinal fate. Targeted co-expression of *dac* and *eya* induces *glass* (a marker of differentiated PRs) expression in almost all tissues that are transformed into retinal fate suggesting that strong synergistic action of *dac* and

eya is sufficient to induce ectopic retinal development in *Drosophila* (Chen et al. 1997). Since *dpp* expression marks the position of MF, targeted co-expression of *dac* and *eya* induces ectopic *dpp* expression in the eye-antennal disc as well as in the leg disc. Dac and Eya both encode nuclear proteins, and by using yeast two-hybrid system and in vitro binding studies, physical interaction between Dac and Eya was confirmed (Chen et al. 1997; Tavsanli et al. 2004). However, RDN genes do not act in a simple, linear pathway but rather are involved in a multiple positive feedback loops during normal eye development. For example, although *dac* acts downstream of *eya*, forced expression of both *dac* and *eya* strongly induces expression of each other. Similarly, since *ey* acts upstream of *eya*, misexpression of *dac* and *eya* is also sufficient to induce ectopic *ey* expression. Additionally, ectopic eyes formed by co-expression of *dac* and *eya* is blocked in ey^2 mutant background suggesting that induction of *ey* expression is essential.

Nmo Interaction with So-Eya Complex Promotes Retinal Specification

Eye-specific *eya* heterozygous mutants have no external phenotype, whereas homozygous mutants are marked by loss of compound eye formation. Interestingly, *eya* heterozygous flies in *nmo* homozygous mutant background show reduction of the ventral eye and eye-to-head transformation. This indicates that *eya* and *nmo* may normally act together during early patterning of the eye (Braid and Verheyen 2008). The role of nmo is further investigated during ectopic eye formation where it is shown that cells that are transformed into eye fate in the head, wing, and leg show ectopic *nmo* expression. Furthermore, since targeted *eya* induce ectopic eye formation, these ectopic eyes were significantly less formed in *nmo* mutant background suggesting that *nmo* may act as a positive component of retinal determination (Braid and Verheyen 2008). *nmo* functions downstream of *eya*, and targeted co-expression of *nmo* and *eya* synergistically enhances the frequency and size of ectopic eyes in the head, wings, and legs. Similar synergy is also observed by targeted co-expression of *nmo* and *dac* (Braid and Verheyen 2008).

Nmo encodes a proline-directed serine/threonine kinase, and results show that synergistic enhancement in the formation of ectopic eyes by targeted co-expression of *eya* and *nmo* requires Nmo kinase domain. Eya directly interacts with So and forms So-Eya complex which is required for downstream transcription of target genes, and it is found that Nmo's kinase activity potentiates So-Eya-mediated induction of the target genes. Nmo may form a molecular complex with Eya and phosphorylates Eya at two conserved MAPK phosphorylation consensus residues to promote activation of So-Eya transcriptional complex during retinal specification (Morillo et al. 2012). It is believed that Nmo and Eya association could be an intrinsic part of the So-Eya transcriptional complex and inclusion of Nmo from that complex would give you a dynamic modulation of the transcriptional output. Mechanistically, Nmo's association with the So-Eya transcriptional complex may occur either by recruitment to DNA-bound So-Eya complex or Nmo itself could

occupy target sites and then recruit So-Eya complex. However, the exact biochemical mechanism is still unknown.

Role of Tsh, Tio, and Hth as Suppressors of Eye Specification

tsh acts as an activator and promotes eye development at the dorsal margin of the eye, whereas it also acts as a repressor and suppresses eye development at the ventral margin of the eye. Targeted *tsh* expression in the eye-antennal disc induces *hth* expression at the transcription level and suppresses eye fate mainly in the ventral region of the eye (Fig. 3). However, *tsh* misexpression in the *hth* mutant background shows significant reduction in the *tsh*-associated eye phenotype (split eye phenotype) suggesting that *tsh*-mediated eye suppression is *hth* dependent. In the developing eye disc of third instar larvae, *ey* expression largely overlaps with *tsh* expression and *hth* overlaps with *tsh* in a three- to four-cell-wide stripe at the anterior end to the MF. *hth* induces a positive feedback loop with *wingless* (*wg*) signaling only at the ventral region of the eye disc, but not at the dorsal region (Pichaud and Casares 2000). Wg signaling also collaborates with *tsh* to induce ectopic *hth* transcription and thereby potentiates *hth*-mediated suppression of eye fate.

tio is a paralog of *tsh* and it is expressed in an identical pattern to *tsh* in the developing retina. Forced expression of *tio* can also induce ectopic eyes in a broader range than *tsh* and is also a more potent inducer of eye formation and tissue growth (Bessa et al. 2009; Datta et al. 2009). Since both *tsh* and *tio* encode zinc-finger transcription factors, deletion of zinc-finger domain hampers ectopic eye formation in case of both *tio* and *tsh* suggesting that zinc-finger domain is required for promoting eye development. Targeted overexpression of both *tsh* and *tio* in the eye, wing, and leg imaginal disc downregulates each other's transcription level suggesting that both *tsh* and *tio* are engaged in a negative feedback loop (Fig. 3). *tsh* gain of function downregulates its own expression level, whereas loss of function upregulates it confirming that *tsh* maintain its level through a negative autoregulatory loop (Bessa et al. 2009).

Loss-of-function clones of the homeobox gene *hth* in the ventral head lead to ectopic eye formation, whereas its ectopic expression in the eye-antennal disc leads to eyeless phenotype suggesting that *hth* acts as a potent repressor of the eye development (Fig. 3). Hth inhibits eye development by disrupting the efficient MF propagation and possibly also MF initiation (Pai et al. 1998). It is reported that in the developing eye disc at larval third instar, *hth* expression is restricted anterior to the MF and also to the dorsal and ventral part of the presumptive head capsule. However, *hth* is required only in the ventral region for *wg* maintenance and for the suppression of eye development. *wg* upregulates *hth* expression in both the dorsal and ventral region of the presumptive head capsule suggesting that both *wg* and *hth* are involved in a positive feedback loop (Pichaud and Casares 2000).

Role of Antennal Gene *dan* and *danr* as Regulators of Eye Specification

dan and *danr* are genes involved mainly during antennal specification, and they are both necessary and sufficient to induce antennal fate (Emerald et al. 2003; Suzanne et al. 2003). However, like other RDN genes, targeted expression of both *dan* and *danr* in the antennal disc is sufficient to transform antennal precursors to an eye fate (Curtiss et al. 2007). Expression of *dan* in the developing eye disc overlaps with *atonal (ato)* expression, and mutational studies by using different *dan* and *danr* alleles show that both *dan* and *danr* are required for ommatidial patterning by positively regulating *ato* and *Egfr* expression in the differentiated PRs (Curtiss et al. 2007). Expression pattern of *dan* and *danr* resembles closely to the expression of *eya*, *so*, and *dac* throughout adult development, and *dan/danr* loss-of-function alleles show that *dan* is required for inducing high levels of *eya* expression. Also, *dan and danr* expression requires *so* activity, and although *dac* is not required for *dan* expression, it initiates *danr* expression. Additionally, misexpression of *dan* or *danr* induces ectopic *ey* expression suggesting that both *dan* and *danr* are also involved in feedback loops that are required to induce RDN gene expression (Curtiss et al. 2007). Furthermore, by using GST pulldown assay and yeast two-hybrid assay, it was confirmed that both Dan and Danr physically interact with itself as well as with each other. Additionally, they also physically interact with Ey and Dac showing a direct involvement of Dan and Danr during retinal determination process (Fig. 3) (Curtiss et al. 2007).

Eye Field Determination and Patterning of Visual Anlage in the Embryo

Specification of precursors that are required to form adult structures starts early in the embryo. Primordial cells that form specific adult tissues are organized in the imaginal discs. Imaginal discs are formed from groups of founder cells located along the anterior-posterior body axis during blastoderm stage in the embryo (Crick and Lawrence 1975; Simcox and Sang 1983). Several adult structures including the adult feeding organs, eyes, antenna, legs, halteres, wings, internal and external genitalia, as well as the epidermis are derived from imaginal discs. However, it remains unknown how and when imaginal disc cells in the embryo are organized to form any particular structures.

At the blastoderm stage, all components of the visual system are coupled to a single unpaired primordium (also considered as nonsegmental acron) located at the dorsal midline in the anterior head region of the embryo. Fate mapping and lineage tracing have revealed that the formation of eye-antennal imaginal disc begins with 5–20 cells, and these cells not only form eye-antennal disc, but they build most of the larval and adult visual system (Jurgens and Hartenstein 1993). However, the eye field that gives rise to eye-antennal disc is initially arranged in an elongated strip in the epidermis where primordia of the presumptive disc form V-shaped structure.

The presumptive eye-antennal disc containing cells is derived mainly from three head segments in the embryo: acron, antennal, and maxillary segments. While the expression of *engrailed* (*en*) marks the segmental subdivision in the embryonic head, zinc-finger transcription factor *escargot* marks the eye primordium (Hartenstein and Jan 1992; Hayashi et al. 1993; Younossi-Hartenstein et al. 1993).

The eye-antennal disc is composed of two opposing epithelial layers: the tall and narrow columnar main epithelium (ME), also known as disc proper, and broad, flattened squamous peripodial epithelium (PE) (Haynie and Bryant 1986; McClure and Schubiger 2005; Atkins and Mardon 2009). These two epithelial layers are separated by narrow margin of cells at the border between ME and PE and are called "cuboidal margin" cells. During the development of eye-antennal disc, the eye part gets derived from the ME, whereas PE contributes to the head capsule surrounding the eye (Bessa and Casares 2005).

Specification and determination of eye-antennal disc occurs during embryogenesis and is developmentally controlled by various intracellular and extracellular cues. It includes *Drosophila Pax-6* gene eyeless (*ey*) and *twin of eyeless* (*toy*), which are evolutionary conserved transcription factors and are both necessary and sufficient to lock the cell toward eye cell fate (Gehring and Ikeo 1999; Gehring 2004; Kozmik 2005). They are expressed in a distinct spatiotemporal pattern and are co-expressed only to the specific subsets of cells of the embryonic brain and presumptive eye-antennal disc primordia (Czerny et al. 1999; Kammermeier et al. 2001). By the end of embryonic stage 15 when eye-antennal disc primordia arise as a result of invagination of cells from the anterior neuroectoderm, *toy* and *ey* start to get expressed in these cells showing a clear epistatic relationship (Czerny et al. 1999). *ey* is first detected during late germband extension at stage 10 in the embryo in every segment of the ventral nerve cord. During subsequent development, its expression marks a broad domain, covering the eye-antennal disc primordia, optic lobe primordia, and other discrete regions of the embryonic brain and ventral nerve cord (Quiring et al. 1994; Halder et al. 1995). Similarly, *toy* expression is first detected at the cellular blastoderm stage, and during subsequent development, its expression domain occupies the dorsolateral head ectoderm that gives rise to the brain and most part of the visual system including the optic lobe as well as eye-antennal disc primordia (Czerny et al. 1999). In the early embryo, *ey* is expressed in an area in the developing ventral nerve cord where *toy* expression is absent, and later during embryogenesis, both genes are expressed in the ventral nervous system in different subset of cells. While *ey* is expressed in few cells of the developing brain, its expression remains regionalized in both brain hemispheres. However, *ey* and *toy* do get expressed in a very similar manner in the developing visual system that includes co-expression of both genes in the optic lobe and eye-antennal disc primordia of the late embryo (Czerny et al. 1999).

During embryogenesis *toy* is expressed earlier than *ey*, and genetic and biochemical studies have demonstrated that both *toy* and *ey* have nonredundant functions during eye development. *toy* acts upstream of *ey* in the genetic cascade, and by directly binding and regulating the ey enhancer, it activates *ey* expression and promotes the onset of eye developmental program in the embryo. However, in order to understand specification of embryonic eye anlage, it is critical to understand how

toy gets activated in the *Drosophila* embryo. *toy* activation occurs during cellular blastoderm stage by the maternally provided transcription factors and gap gene proteins that initiate embryonic patterning along the anterior/posterior (A/P) and dorsal/ventral (D/V) axis. After fertilization, the maternally contributed *bicoid* (*bcd*) mRNA is translated and organizes the anterior embryonic patterning by forming a gradient of Bcd protein at the A/P axis with its highest peak at the anterior pole. *torso* (*tor*) which encodes a maternally expressed tyrosine kinase receptor is although uniformly distributed in the blastoderm membrane but only gets activated at the poles by a localized ligand (Duffy and Perrimon 1994). While *tor* domain of action is particularly restricted to the poles, *tor* loss-of-function embryos show loss of *toy* expression, whereas *tor* gain-of-function shows broader *toy* expression. Therefore, it is synergistic association of anterior and posterior system that is required for *toy* expression at the blastoderm stage. The anterior-posterior gradient of *bcd* triggers the transformation of maternal *hunchback (hb)* which is transiently present in the anterior half of the embryo to the transcriptionally active zygotic *hb* (Driever and Nusslein-Volhard 1988). Activation of *hb* prevents premature activation of *toy*. At mid-cellular blastoderm stage, when *hb* expression gets retracted from the anterior pole (Ronchi et al. 1993). *toy* expression was further prevented by the expression of *knirps* (*kni*) which is activated by joint action of Bcd and Dorsal (Dl) (Rothe et al. 1994). Dl is a maternally contributed transcription factor that forms dorsoventral concentration gradient and this gradient is responsible for patterning the embryo at the D/V axis (Courey and Huang 1995). At the late cellular blastoderm stage, hb domain gets resolved into two anterior stripes (cephalic and parasegment) and *toy* gets transcribed particularly in the cephalic region of the embryo by the combined action of Bcd, Tor and Dl. Therefore, Dl is one of the key molecular player involved with dual effect to regulate *toy* expression: negative effect mediated by *kni* and a positive effect together with *bcd* and *tor*. However, the positive effect of Dl is limited and it is synergistic association of *bcd* and *tor* which is necessary for *toy* transcription.

Specification of Eye During Eye-Antennal Disc Development at Different Larval Stages

Specification of eye from the developing eye-antennal disc in *Drosophila* begins with small group of cells that were left-aside during embryogenesis. After the embryo hatches, cells of the presumptive eye-antennal disc primordia undergo continuous proliferation. During late second instar larval stage, tissue-specific gene regulatory networks are expressed within distinct eye and antennal field and initiate subdivision of the eye-antennal disc primordia. In the eye field, RDN genes are the ones that form tissue-specific gene regulatory network and control eye development. Growth and development of the disc is further controlled by interplay of transcriptional determinants involved in the RDN with extracellular signaling pathways. Lastly, maintenance of the eye and antennal fate after establishment of eye and

antennal primordia is further controlled by mutual repression (by antagonizing each other). In this part we will review how developmental plasticity of the eye-antennal disc is maintained till second instar larval stage. We will also discuss how onset of key RDN gene expression triggers the eye fate and how extracellular signaling molecules interplay with transcriptional determinants to segregate eye and antennal fate. Lastly we will also review how eye and antennal fates are maintained during later developmental stages by mutual antagonistic repression.

First and Second Instar Larval Stage Shows Developmental Plasticity in the Eye-Antennal Disc

The process of regional specification during development of eye-antennal disc primordium begins with a group of uniform cell population that acquires distinct cellular identity by forming tissue-specific domains and sub-domains. In *Drosophila* as well as in vertebrates, this process of regional specification is controlled by both key transcriptional regulators encoded by "selector genes" and conserved signaling molecules that are repeatedly utilized throughout development (Mann and Morata 2000; Curtiss et al. 2002). The development of eye-antennal disc that gives rise to distinct adult structures (eye, antenna, ocelli, palpus, and the surrounding head cuticle) starts from distinct number of cells that are set aside during embryogenesis (Garcia-Bellido and Merriam 1969; Wieschaus and Gehring 1976; Mandaravally Madhavan and Schneiderman 1977). However, the segregation of eye and the antennal fate is missing in the embryo which can be correlated with the lack of essential factors required for the determination of eye and antennal primordia. At the first instar larval stage, eye-antennal epithelium consists of a small cluster of cells with little or no evidence of regional patterning. During the first and second instar larval stage, cells of the eye-antennal disc divide and grow to form pool of progenitor cells required for adult head structures. The earliest indication of regional identity of eye and the antennal field appears during mid- to late second instar larvae. The eye field grows and forms eye proper, head cuticle, and the ocelli, whereas antennal field grows and forms antenna and the head cuticle (Haynie and Bryant 1986).

Onset of Eye and Antenna-Specific Transcription Factor's Expression During Eye-Antennal Fate Segregation

Initiation of gene expression and the formation of tissue-specific gene regulatory network required for eye-antennal fate segregation begin from mid- to late second instar larval stage. Several genes that regulate specification of the retina, antenna, and surrounding head capsules are expressed throughout the entire eye-antennal disc primordium (Quiring et al. 1994; Royet and Finkelstein 1996; Czerny et al. 1999; Kumar and Moses 2001a, b; Aldaz et al. 2003; Jang et al. 2003). For example,

ey and *toy* which are core members of the RDN and their expression in turn activates other genes of the network are expressed in the embryo and during first instar in the entire eye-antennal disc. Simultaneous removal of *ey* and *toy* early when they are universally expressed in the eye-antennal disc hampers the formation of entire disc and its associated head structures. In addition to *ey* and *toy*, two additional *Pax6*-related genes *eyg* and *toe* are also expressed in the embryonic eye-antennal disc primordia (Jun et al. 1998; Aldaz et al. 2003; Jang et al. 2003). However, their expression is turned off during larval first instar, and it gets reinitiated during late larval second instar in the dorsoventral region of the disc (Chao et al. 2004; Dominguez et al. 2004). Although *ey* acts downstream of *toy*, it acts as a master regulator that controls, either directly or indirectly, expression of other RDN genes to execute the eye program. Similarly, *hth*, *exd*, *dll*, and *cut* act as selectors for antennal development and are individually required for the development of the antenna. During late second instar larval stage, *ey/toy* expression is restricted to the posterior two-thirds of the disc, whereas homeodomain transcription factor *cut* is turned on in the anterior third of the disc. *ey/toy* and *cut* expression domains mark the territories of the future eye and antenna, respectively. Another homeodomain encoding transcription factor *distalles* (*dll*) within the *cut*-expressing domain co-expresses with *hth*, and together they specify the antennal fate (Casares and Mann 1998; Dong et al. 2000). *hth* and *exd* both are expressed in the eye and antennal region of the eye-antennal disc and serve as negative regulators of the eye development by delimiting the eye field during adult head development in order to prevent inappropriate eye formation (Pai et al. 1998; Bessa et al. 2002). During eye-antennal fate segregation at the late second instar larval stage, *eya* expression starts at the posterior margin of the eye disc followed by *so* and *dac* expression (Kenyon et al. 2003). Since these genes are required for the initiation of eye differentiation process, it was proposed that expression of these genes in the late second instar larval stage locks the identity of cells to attain retinal fate (Kumar and Moses 2001a).

Role of Extracellular Signaling Pathways in Segregating Eye and Antennal Fate

While RDN functions as a unit to promote retina development, it also integrates instructions that are transmitted across the developing eye-antennal disc by diffusible morphogens and signal transduction pathways. These signaling cascades are repeatedly used in a spatiotemporal manner during eye development and intersect RDN at multiple levels (Kumar 2001; Voas and Rebay 2004). Some of these include the Notch, EGFR, Dpp, Hh, and Wingless signaling pathway which are described in this section below.

Proliferative growth is a dominant feature during the development of eye-antennal disc from first instar to late second/early third instar larval stage, and this continuous proliferative signal is provided by Notch signaling pathway. Although

Notch receptor is expressed ubiquitously in the entire eye-antennal disc, activation of Notch signaling pathway only occurs along the dorsoventral compartment boundary of the eye disc by binding the Notch receptor with its ligands Delta and Serrate (Cho and Choi 1998; Dominguez and de Celis 1998; Papayannopoulos et al. 1998). In addition to setting up the dorsoventral compartmental boundary, Notch is also involved in establishing planner polarity, spacing of ommatidial clusters, and cell fate specification (Cagan and Ready 1989b; Blair 1999; Baker 2000). Notch mediates eye growth by regulating the expression of *eyg* in the dorsoventral region of the second instar eye disc (Chao et al. 2004; Dominguez et al. 2004; Rodrigues and Moses 2004). *eyg* belongs to the *Pax6(5a)* family, and following its consistent role in growth control, human *pax6(5a)* cDNA is expressed in the *Drosophila* imaginal disc which results in the massive overgrowth phenotype (Dominguez et al. 2004). Furthermore, *unpaired* (*upd*) is identified as the ligand of JAK/STAT pathway, and since it acts as the target of Eyg, it may act over long distances to promote Notch-Eyg-mediated eye disc growth (Fig. 4) (Chao et al. 2004).

The *Drosophila* EGF receptor homolog (Egfr) is a transmembrane receptor tyrosine kinase. Removing *Egfr* function in the eye-antennal disc leads to complete loss of eye and antenna in the eclosed adults indicating that Egfr is required during both

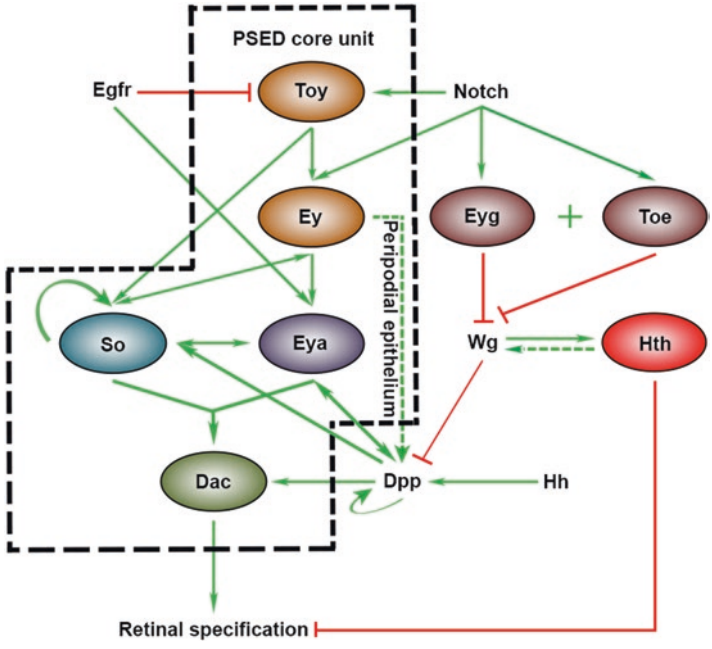

Fig. 4 Interplay of RDN genes with the extracellular signaling pathways. Schematic representation of all known interactions of RDN genes with the extracellular signaling pathways mediating retinal specification during eye imaginal disc development. PSED (Pax-Six-Eya-Dac) core unit represents important members of the RDN, which are crucial for regulating overall size of the compound eye. Additionally, in the peripodial epithelium, Ey activates Dpp signaling and promotes retinal specification. Green arrows represent activation, whereas red lines show repression (Modified from Kumar 2009a; Baker et al. 2018)

eye and antennal specification, determination, and survival (Bergmann et al. 1998; Freeman 1998; Kurada and White 1998; Kumar and Moses 2000). Egfr ligand Spitz (only secreted form of Spitz) is sufficient for the homeotic transformation of the eye to the antenna, and increasing Egfr activity further strengthens the homeotic transformation phenotype suggesting that Egfr signaling is required for the maintenance of eye and antennal identity (Kumar and Moses 2001a).

During the development of eye-antennal disc, it is quite intriguing to see how early RDN genes such as *ey* and *toy* are initially transcribed in the entire eye-antennal epithelium and later how their expression pattern becomes restricted only to the presumptive eye region. It appears that Notch signaling plays an important role here, and it both activates and maintains *ey* expression (Fig. 4), whereas in the second instar larval stage, Egfr pathway antagonizes Notch signaling by restricting *ey* transcription in the developing eye (Kurata et al. 2000; Kumar and Moses 2001a). Egfr activity is also antagonized by Notch signaling during cell fate decisions in the fly eye (Fortini et al. 1993; Sawamoto and Okano 1996), and it is phenotypically apparent by transformation of the eye to antennal fate upon expression of dominant negative transgenes of the *Delta* or *Serrate* (Kumar and Moses 2001a). However, during the development of MF, Notch and Egfr do not antagonize each other, but they are both required for the initiation and progression of the MF (Kumar and Moses 2001b). Further epistatic experiment shows that during eye-to-antennal transformation, transcription levels of key RDN genes (*ey*, *toy*, *eya*, *so*, and *eyg*) are significantly low suggesting that both Egfr and Notch signaling pathways genetically act upstream to the eye specification genes (Fig. 4).

Superfamily of TGF-ß signaling pathway controls diverse processes during development of both vertebrates and invertebrates that include establishment of body axes, cell proliferation and death, and cell fate determination and differentiation (Kingsley 1994; Hogan 1996). The *Drosophila* TGF-ß homolog *decapentaplegic* (*dpp*) play essential roles during both embryonic and larval developments that include establishment of dorsoventral polarity, midgut formation, proliferation and patterning of the larval imaginal discs, and primordia of the adult tissues. At the second instar larval stage, *dpp* is expressed toward the posterior and lateral margin of the eye disc, and at the early third instar when ommatidial differentiation begins, *dpp* expression is localized to the MF (Blackman et al. 1991; Pignoni and Zipursky 1997). Experiments on hypomorphic eye-specific $dpp^{d\text{-}blk}$ allele and analysis of *Mothers against dpp (Mad) mutants*, which functions downstream of *dpp*, show that *dpp* is involved in the initiation of the MF (Treisman and Rubin 1995; Newfeld et al. 1996; Wiersdorff et al. 1996). Ectopic expression of *dpp* is sufficient to induce ectopic MF initiation and eye disc duplication selectively at the anterior margin of the eye disc (Pignoni and Zipursky 1997). Since proliferation alone is not sufficient to generate an eye disc, *dpp* may act with other factors during growth and differentiation in a way that fully recapitulates the wild-type eye disc development program. It was shown that *dpp* signal is required for the initiation of key RDN genes such as *so*, *eya*, and *dac* in the developing eye disc (Fig. 4) but is not required for their maintenance (Curtiss and Mlodzik 2000). One recent study suggests that no-eyed phenotype caused by mutations in the core PSED (Pax-Six-Eya-Dac) unit of the RDN can

be rescued by simply restoring Dpp back to the eye field (Fig. 4) (Baker et al. 2018). This observation is further supported by data showing that ectopic eyes induced by *ey* occur mainly at places where normal *dpp* expression was observed implicating that *dpp* is a key downstream target molecule for eye specification process. *dpp* positively autoregulates its own expression since ectopic *dpp* is sufficient to broaden *dpp* expression domain and therefore in the second instar *dpp* induces proliferation to broaden Dpp domain and as a result initiates eye development at the anterior site.

Hedgehog (Hh) is a secreted morphogen of the Hh signaling pathway which is autoproteolyzed to form N-terminal fragment, and this fragment serves as a signaling ligand (Lee et al. 1994; Porter et al. 1995). Hh binds to its receptor Patched (Ptc), and this binding activates hyperphosphorylated Smoothened (Smo) that further activates the transcription *factor cubitus interruptus* (*ci*). Activation of *ci* induces transcription of downstream target genes like *dpp* and *ptc* (Ingham 1998; Aza-Blanc and Kornberg 1999; Ingham et al. 2000; Ingham and McMahon 2001). Ectopic *dpp* induces the same effect as it is seen with ectopic *hh* clones indicating that primary function of Hh is to precisely control expression of *dpp* within Dpp expression domain (Zecca et al. 1995). Hh is secreted by the differentiating neurons at the posterior side to the MF, and eye disc in *hh* mutants is associated with significant reduction or elimination of *dpp* expression. Additionally, ectopic Hh induces *dpp* expression, MF formation, as well as ommatidial differentiation suggesting that *hh* is required to turn on *dpp* expression (Fig. 4) and MF progression across the eye disc (Heberlein et al. 1993; Ma et al. 1993). Conversely, Hh receptor Patched (Ptc) and protein kinase A (PKa-C1) act as negative regulators of the *dpp* expression and the eye development. However, no direct interaction of Hh and Dpp signaling was observed, and therefore antagonistic effect of Hh and Dpp during the formation of visual structure is most probably based on an indirect interaction between these two morphogens (Chang et al. 2001). In the developing eye-antennal disc, *ey* is expressed initially in the entire eye disc, and later its expression is restricted anterior to the MF (Halder et al. 1998). It is shown that although *hh* is required for *ey* expression in the embryonic eye primordium, Hh and Dpp signaling is not required for *ey* expression in the eye disc (Chen et al. 1999; Curtiss and Mlodzik 2000; Chang et al. 2001). Epistatic relationship of Ey with Hh and Dpp signaling was confirmed by experiments that show requirement of high Hh and Dpp signals during Ey-mediated induction of ectopic eyes, and these ectopic eyes failed to induce in *hh* mutant flies (Kango-Singh et al. 2003).

wingless (*wg*) is the founding member of Wnt family and acts as a morphogen that has multiple roles during eye development. Since developing eye disc is subdivided to give rise to the retina and head capsule, Wg signaling plays a critical role here to promote head capsule formation at the expense of retina development. Reduction in the activity of Wg signaling promotes eye field expansion into the lateral region of the dorsal head, whereas expression of negative regulators of Wg signaling such as *shaggy* (*sgg*) or *axin* (*axn*) promotes the transformation of eye field into the head cuticle (Baonza and Freeman 2002). Wg is expressed throughout the entire second instar eye disc where it blocks expression of *eya* by blocking Dpp signaling (Fig. 4) (Royet and Finkelstein 1997). In the larval third instar, Wg

expression gets restricted to the anterior lateral margin to the MF where it represses the expression of early RDN genes such as *eya*, *so*, and *dac* in conjunction with *tsh* to promote the formation of dorsal head (Pignoni and Zipursky 1997; Baonza and Freeman 2002). Also, since *hth* acts as a known repressor of the PR differentiation in the eye, Wg may promote head fate by inducing the expression of *hth* (Fig. 4) (Pichaud and Casares 2000). Wg signaling is also regulated by JAK/STAT signaling pathway where the ligand *upd* is expressed at the posterior margin of the eye disc and promotes the formation of eye field by repressing *wg* transcription (Ekas et al. 2006). Therefore, during eye disc development, subdivision of the head field versus eye field is determined by negative interaction between anteriorly expressed Wg signaling and posteriorly expressed Hh, Dpp, and Upd signaling (Baonza and Freeman 2002; Legent and Treisman 2008). However, overlaps between these signals during eye disc development are separated by growth of the eye disc controlled by Notch signaling pathway (Dominguez et al. 2004). Additionally, Wg signaling is also involved during the growth of eye disc since temperature-sensitive alleles show small eye discs, whereas overexpression of Wg results in the dramatic overgrowth phenotype (Treisman and Rubin 1995; Baonza and Freeman 2002).

Initiation and progression of MF require positive regulation by Hh and Dpp signaling, whereas Wg signal acts as a negative regulator. Notch cooperates with Egfr signaling and promotes initiation of the furrow. Epistatic experiment shows that when Wg was co-expressed with Egfr, induction of ectopic furrows occurs suggesting that Wg signal acts upstream of the Egfr signaling pathway. Hh and Dpp signaling induces MF formation at the posterior margin (Heberlein and Moses 1995). However, it is not clear which is upstream of the other's during eye development process. Notch signal blocks furrow initiation, whereas Egfr signaling is involved in the initiation of the MF, and epistatic experiment shows that Notch acts downstream of the Egfr signaling pathway during eye development process. Early retinal gene activation requires Dpp signaling, whereas high Wg expression blocks this activation (Chanut and Heberlein 1997; Hazelett et al. 1998; Chen et al. 1999; Curtiss and Mlodzik 2000; Kenyon et al. 2003). Two alternate models were proposed to explain events during early eye specification. According to the first model when Wg signals are high during second larval instar, no initiation of eye specification takes place. However, growth of the eye disc induced by Notch signaling pathway separates Wg and Dpp expression domain, and due to this separation, Wg signal goes down from the posterior cells. The immediate effect of low Wg and high Dpp in the posterior region results in clearance of *hth* which is under Wg control and early expression of retinal genes which was suppressed due to high Wg expression (Dominguez and Casares 2005). According to the second model, growth of the eye disc by Notch signaling enables anterior "head" region (marked by *hth* expression) and posterior "eye" region (marked by *eya* expression) in response to Wg and Dpp signaling, respectively. During the disc growth, opposing domains marked by *hth* and *eya* act as a trigger to promote initiation of the retinal differentiation process by initiating *eya-so and dac* complex (Dominguez and Casares 2005).

Maintenance of Eye and Antennal Fate by Mutual Repression

It is quite intriguing to see how adjacent primordia specify and attain different developmental fates and how these fates are stably maintained during development. For example, most adult head structures of *Drosophila* originate from bilaterally symmetric group of cells in the embryonic head region called as eye-antennal disc primordium. Cells in the primordium proliferate during different larval stages to form eye-antennal imaginal disc. Segregation of eye-antennal disc forms eye disc that later developed into compound eyes and ocelli in adults and antennal disc that form antenna and maxillary palp in adults.

The segregation of eye and antennal primordia is maintained by mutual repression of eye- and antenna-specific genes, and they mutually antagonize each other to maintain two developmental fates during eye-antennal imaginal disc development (Wang and Sun 2012). For example, early eye-antennal disc expresses *ey* which is required for the eye development (Quiring et al. 1994). *ey* directly activates *so* expression and thereby triggers eye specification process. Both *ey* and *so* are uniformly expressed in the eye-antennal disc at first instar larval stage, whereas their expression becomes restricted to the eye part during second instar larval stage. Similarly, *hth* is uniformly expressed in the early eye-antennal disc, whereas its expression is retracted from the posterior region of the eye disc, and at the third instar, *hth* is expressed in the proximal region of the antenna disc (Casares and Mann 1998; Pai et al. 1998). In the eye disc, *hth* expression is divided into two regions: anterior region where *ey* is not expressed and *hth* which blocks eye development (Pai et al. 1998) and posterior region where *hth* is co-expressed with *ey* and involved in the maintenance of proliferation and keeping cells at undifferentiated state (Bessa et al. 2002). *cut* is expressed in the antenna disc and works redundantly with *hth* to repress retinal development pathway by repressing *ey* transcription. Similarly, *so* represses *cut* and *hth* expression and maintains eye fate (Wang and Sun 2012). During eye-antennal disc development, loss of *cut* and *hth* from the antennal disc induces antenna-to-eye transformation, whereas loss of *so* in the eye disc induces eye-to-antennal transformation (Wang and Sun 2012). Therefore, a way to achieve stable fate maintenance during segregation of eye and antennal region in the developing eye-antennal disc is mutual antagonism between genes involved in eye or antenna specification.

Role of RDN During Development of Extraretinal PRs in *Drosophila*

Role of RDN During Larval Eye Development

All visual organs in *Drosophila* develop from optic placode which is formed in the dorsolateral region of the head ectoderm in embryos (Campos-Ortega and Hartenstein 1985). During embryonic development, neuroepithelial cells of the

optic placode are arranged to form two domains: the ventroposterior domain which forms larval eye primordium and the dorsolateral domain that generates eye-antennal imaginal disc and optic lobe primordium of the adult visual system (Hartenstein 1988; Daniel et al. 1999; Suzuki and Saigo 2000). The larval visual organ (also called Bolwig's organ) is composed of two bilaterally positioned bundles of 12 PR cells (Bolwig 1946; Serikaku and O'Tousa 1994). Since both larval and adult visual systems originate from the same ectodermal invagination in the embryo (Green et al. 1993), it was proposed that the developmental mechanisms regulating initial specification of both larval and adult visual systems must have some overlapping features.

The development of adult visual system has been studied in great detail, and it is known that RDN genes play important roles in the initiation and maintenance of the eye fate during eye-antennal disc development. Comparatively, larval eye development begins after optic placode invagination from the embryonic ectoderm at stage 12 (Green et al. 1993). The earliest known RDN gene is *toy* which is expressed in the embryonic primordia of the larval eye at the posterior procephalic region (Czerny et al. 1999). However, in the differentiating cells of the larval eye later during embryogenesis, *toy* expression was absent. Expression of *ey* looks very similar, if not identical, to that of *toy* in the developing visual system in embryos except the region where larval eye primordium is formed (Czerny et al. 1999). Since both *ey* and *toy* are capable of initiating eye developmental program (Halder et al. 1995; Czerny et al. 1999), neither *ey* nor *toy* is required for the development of larval visual system (Suzuki and Saigo 2000). Another early RDN gene *so* is expressed early in the embryonic optic placode region and is subsequently expressed in the optic placode throughout embryogenesis. In the *so* null mutant embryos, optic placode failed to invaginate, and it leads to the absence of the larval eye suggesting that *so* is critical for the formation of larval visual organ (Cheyette et al. 1994). It is also known that So interacts directly with Eya and form a complex which is required for the initiation of eye specification during eye-antennal disc development (Pignoni et al. 1997). *eya* is expressed in an identical manner as *so* and *eya* null mutant embryos show phenotype resembling *so* mutant phenotype in terms of absence of the larval eye (Suzuki and Saigo 2000). During larval eye development, So forms a direct complex with Eya and is required for the initiation of *atonal (ato)* expression. *ato* is a basic helix-loop-helix transcription factor required for the compound eye development (Jarman et al. 1994) and is one of the first factors which is expressed in the differentiating cells of eye during eye-antennal imaginal disc development. Similarly, *ato* expression was also found in the presumptive larval eye in the embryo (Daniel et al. 1999; Mishra et al. 2018) where it acts as a determinant of larval PR formation and is regulated by the activity of both *so* and *eya* (Suzuki and Saigo 2000).

In summary, members of the RDN gene family are not only involved during adult visual system development but are also required for the development of extraretinal larval visual system in *Drosophila*.

Role of RDN During Adult Ocelli Development

Invertebrate visual system is a fine example to show how different light-sensing structures accommodate broad range of functions. In *Drosophila*, for example, compound eyes are mainly associated with motion detection and color or polarized vision in adults, whereas ocelli appeared to be involved mainly during flight by sensing gravity and balancing the body by detecting changes in the light intensity (Hu et al. 1978; Hu and Stark 1980; Hardie 1985; O'Tousa et al. 1985; Zuker et al. 1985; Pollock and Benzer 1988; Yoon et al. 1996; Gao et al. 2008; Krapp 2009; Yamaguchi et al. 2010). Ocelli in *Drosophila* are three simple eyes (one anterior or medial and two posterior or lateral) that are arranged in a triangular shape between two compound eyes at the vertices on the dorsal head. Both compound eye and ocelli are derived from the eye-antennal imaginal disc of third instar larvae. While the compound eye is derived from central part of the eye morphogenetic field, ocelli are derived from two clusters of cells in the anterior dorsal part of the eye field (Royet and Finkelstein 1996).

Despite morphological disparity existed between compound eye and ocelli, profound homologies exist in terms of the genetic program that control development of both organs. A group of evolutionary conserved genes collectively known as RDN genes are involved in the determination of both compound eye and ocellar primordia during eye-antennal disc development (Pappu and Mardon 2004; Silver and Rebay 2005; Kumar 2009a). The *Pax6* homologs *ey* and *toy* are placed at the top of RDN, and together they initiate eye specification process by activating downstream genes of the eye specification cascade that includes activation of *eya* and *so* expression (Bonini et al. 1993; Cheyette et al. 1994; Gehring and Ikeo 1999). *eya* and *so* expression marks two different domains of the eye morphogenetic field, labeling presumptive compound eye and ocellar primordia. The regulation of *eya* and *so* expression in the compound eye is different than in ocelli, and it further depends on *ey* and *toy* distribution in the morphogenetic field of third instar larvae. *ey* is expressed in the compound eye primordium where it activates *eya* and *so* expression (Halder et al. 1998; Zimmerman et al. 2000), whereas *ey* expression is absent in the ocellar primordium. *ey* loss-of-function mutations therefore impair compound eye formation, whereas ocelli are normally formed in this condition (Punzo et al. 2002; Gehring and Seimiya 2010). Conversely, *toy* is expressed in both the compound eye and ocellar primordia. However, function of *toy* during compound eye formation requires the activity of *ey* (Punzo et al. 2002). *toy* loss-of-function mutants are able to form eyes sometimes, but the ocelli are always missing (Jacobsson et al. 2009). *toy* directly initiates the expression of *so* in the ocellar primordium, and proper So protein levels in the disc are maintained through a positive autoregulatory loop mediated by Eya (Pauli et al. 2005). Although *so* expression in the ocellar primordia is *toy*-dependent, *eya* expression seems to be *toy*-independent, but indirectly regulated by a homeodomain containing transcription factor *orthodenticle* (*otd*) (Blanco et al. 2009). *otd* belongs to the conserved *otd/Otx* gene family that is required for the development of the head in both invertebrates and vertebrates (Finkelstein et al.

1990; Simeone et al. 1993; Royet and Finkelstein 1995). During eye-antennal disc development, *otd* is required for the development of head vertex primordium that also includes ocelli (Blanco et al. 2009), and therefore viable *otd* loss-of-function mutants are ocelliless (Finkelstein et al. 1990). *otd* appears to be regulated via two distinct mechanisms. The first mechanism comprises Wg and Hh signaling that may initiate *otd* expression, and the second mechanism comprises a positive autoregulatory loop which maintains subsequent *otd* expression (Blanco et al. 2009).

Extensive genetic interaction analysis has proposed that ocelli development is carried out by two independent regulatory pathways (one controlled by *toy* and the other one controlled by *otd*). These two independent pathways synergistically activate *eya* expression in the ocellar primordium. *eya* activation would in turn trigger *so* expression in the ocellar precursors by an unknown mechanism and maintains *so* expression level. *eya* expression is significantly reduced in *toy* loss-of-function mutants but sufficient enough to activate *so* expression to the wild-type level, and as a result it gives rise to normal flies. However, in few cases, reduction in *eya* activity in *toy* loss-of-function mutants goes beyond a certain threshold that hampers *so* expression in the ocellar precursors, and as a result it gives rise to ocelliless flies (Blanco et al. 2010). In case of *otd* loss-of-function mutants, *wg* is ectopically activated that prevents *eya* and *so* expression in the ocellar primordium, and as a result it gives rise to the ocelliless flies (Royet and Finkelstein 1997; Lee and Treisman 2001; Baonza and Freeman 2002; Blanco et al. 2009).

eya and *so* expression in the developing eye-antennal disc is regulated by *hth*, a known repressor of the eye which together with *tsh* maintains cells in the undifferentiated state and proliferation of the retinal progenitors. Repression of *hth* activity allows induction of RDN gene expression and differentiation (Pai et al. 1998; Bessa et al. 2002; Lopes and Casares 2010). *hth* is initially expressed in the entire ocellar primordium but later gets downregulated by *hh* signaling suggesting that spatial control of *hth* expression is critical for the determination of size of the ocelli and ocellar region.

Concluding Remarks

It is quite clear from all the supporting evidence to date that eye specification cascade is an evolutionary conserved unit that is functional in all seeing animals including humans (Gaspar et al. 2018). Eye development became an area of interest ever since *ey* in *Drosophila* was shown to be homologous to the human *Pax6* gene (Quiring et al. 1994). In particular, since it was shown that *Pax6* is interchangeable across animal kingdom and its misexpression is sufficient to induce ectopic eyes in non-retinal tissues made the RDN an excellent case to study conservation of organogenesis and how transcription factor controls cell fate decision (Halder et al. 1995; Chow et al. 1999). This finding established the view that Ey/Pax6 is the "master regulator" to control eye development process. As a master regulator, the prevailing view was that absence of Ey results in the disruption of gene regulatory network

blocking the compound eye formation. However, presence of retinal tissues in several *ey* mutants further established the view that in the absence of *ey*, a second *Pax6* gene *toy* is able to partially rescue the eye development by weakly activating downstream targets of Ey (Baker et al. 2018). However, it is surprising to see that Toy alone is incapable to substitute role of Ey during eye development. Since Ey and Toy resulted from a gene duplication, if one combines Ey and Toy action, the mode of eye development in *Drosophila* might appear more in line with vertebrates that have only one *Pax6* gene. Indeed, it was shown recently that Ey and Toy should be considered together to regulate similar functions like vertebrate *Pax6* during eye development (Baker et al. 2018). The retina has been used as a powerful model to understand numerous critical biological processes, including programmed cell death, cell proliferation, differentiation, cell cycle control, neuronal development, as well as tissue patterning (Das et al. 2002; Yu et al. 2002; Domingos et al. 2004; Shimamura et al. 2014; Tanaka et al. 2019). Several retinal disorders in human can be directly correlated with mutations in the vertebrate homologs of the eye determination genes and signaling pathways that regulate them. For example, nearly 300 dominant mutations in the human *PAX6* locus have been identified, and most of these mutations lead to a condition called aniridia (Glaser et al. 1992; Jordan et al. 1992; Hanson et al. 1994; Verbakel et al. 2018). Other mutations in *Pax6* or other members of RDN cause spectrum of diseases, and many of them are not well characterized. For example, anophthalmia and microphthalmia have not been well characterized partly because of the polygenic nature of eye development. *Drosophila* provides an excellent system for screening of new candidates in order to improve diagnosis and to understand eye development process in great detail (Wangler et al. 2017; Gaspar et al. 2018). Recently, an elegant screen was conducted to understand sensory functions in *Drosophila* to identify genes that are homozygous lethal. This screening was facilitated by use of sophisticated genetic and molecular tools in order to understand mechanisms underlying human diseases (Yamamoto et al. 2014). However, despite the progress that has been made over the past decade to understand how early decisions are made during the process of retinal determination, still many questions remain unresolved. The advent of new techniques and high-throughput assays such as single-cell RNA sequencing (scRNA-seq) offers the opportunity to detect changes at the cellular level that will provide new insights to understand retinal determination process in great detail. scRNA-seq has been recently used to characterize a transcriptional switch during PR differentiation in the *Drosophila* eye (Ariss et al. 2018). This single-cell atlas of the developing eye can be useful in many ways, such as to decipher developmental trajectory in time, to organize expression domains of genes to unique cell clusters during different developmental stages, and to identify novel genes that will be associated with a specific cell population during eye developmental process. Chromatin immunoprecipitation coupled with next-generation sequencing (ChIP-seq) is also an additional molecular technique that will be extremely useful to find RDN gene interactions with their target genes and pathways in the whole genome during retina development. By using this integrative genomic analysis, putative direct downstream genes that mediate eye specification, differentiation, and patterning can be identified.

Recently, ChIP-seq has been implicated to show *ey* regulation by multiple molecular mechanisms to control target gene expression and pathways during *Drosophila* eye development (Yeung et al. 2018). Additionally, Gal4/UAS system that is routinely used in *Drosophila* to understand gene's function in a spatiotemporal manner (Brand and Perrimon 1993; Blair 2003; Tare et al. 2013) can also be useful for screening of genes involved during eye determination process in a specific time window of development. A collection of transgenic Gal4 lines made at the Janelia farm (Pfeiffer et al. 2008) can be used for this purpose, and recently this tool has been used for the characterization of morphogenetic furrow during patterning and development of the *Drosophila* eye (Sarkar et al. 2018). In the future, development of advanced genetic, molecular, and biochemical tools will provide novel insights to understand retinal determination process in great detail. The mystery of how gene regulatory network controls and coordinates different fates from a large symmetrical population of undifferentiated cells to produce a unique organ or tissue will surely be unrevealed.

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Generation of Third Dimension: Axial Patterning in the Developing *Drosophila* Eye

Neha Gogia, Oorvashi Roy Puli, Akanksha Raj, and Amit Singh

The hallmark of organogenesis in all multi-cellular organisms is transition of the organ primordium cells into a three-dimensional adult organ comprising of three germ layers—ectoderm, mesoderm, and endoderm. Most tissues are derived from epithelial cell sheets, which form highly organized structures. These structures exhibit polarization of apical-baso-lateral axes along with the planar polarity. During organogenesis, many genetically programmed events that are sensitive to environmental cues play major roles. Various models like yeast (*Saccharomyces cerevisiae*), worm (*Caenorhabditis elegans*), fruit fly (*Drosophila melanogaster*), newts (*Notophthalmus viridescens*), mouse (*Mus musculus*), rabbit (*Oryctolagus cuniculus*), guinea pig (*Cavia porcellus*), *etc.* are being used to understand the genetic basis of organogenesis, growth, and regeneration (Mehta and Singh 2019; Singh et al. 2005a, b, 2012; Singh and Irvine 2012). Studies in different model systems have revealed that process of organogenesis involves important events of specification, determination, and differentiation. Any deviation in these events can impair the processes of axes specification, cell proliferation, cell death, and cell differentiation. These cell biological processes work in tandem like part of a genetic orchestra, which results in final sculpting of the organ. Any perturbation in these

N. Gogia · O. R. Puli · A. Raj
Department of Biology, University of Dayton, Dayton, OH, USA

A. Singh (✉)
Department of Biology, University of Dayton, Dayton, OH, USA

Premedical Program, University of Dayton, Dayton, OH, USA

Center for Tissue Regeneration and Engineering at Dayton (TREND), University of Dayton, Dayton, OH, USA

The Integrative Science and Engineering Center, University of Dayton, Dayton, OH, USA

Center for Genome Advocacy (TCGA), Indiana State University, Terre Haute, IN, USA
e-mail: asingh1@udayton.edu

A. Singh, M. Kango-Singh (eds.), *Molecular Genetics of Axial Patterning, Growth and Disease in Drosophila Eye*,
https://doi.org/10.1007/978-3-030-42246-2_2

processes leads to growth and patterning defects in the organs. During organogenesis, the determination of antero-posterior (AP), dorso-ventral (DV), and proximo-distal (PD) axis is referred to as axial patterning. We will focus on contributions from the *Drosophila* eye model to understand these important questions of developmental biology.

Axial Patterning is Required to Generate Three-Dimensional Organ

Organogenesis is a highly complex process, which requires a crucial event of axial patterning. Axial patterning, a lineage restriction event, involves delineation of three different axes, *viz.,* (1) dorso-ventral (DV), (2) proximo-distal (PD), and (3) anterior-posterior (AP) axes (Cohen et al. 1993; Cohen 1993; Held 2002a; Tare et al. 2013a). Any deviation in generation of these axes during eye development results in birth defects like "no-eye" or "reduced-eye" phenotype. These domains are an outcome of progressive restriction of cell fates due to subdivision of the developing field into smaller fields with a more or less rigid developmental potential, which are referred to as the compartments (Blair 2001; Curtiss et al. 2002; Dahmann et al. 2011; Held 2002b; Singh et al. 2012; Tare et al. 2013a). Thus, compartments are basic building blocks formed within a bigger developing field (Blair 2001; Curtiss et al. 2002). The compartment boundaries are defined by the spatio-temporal expression or function of the fate selector genes (Blair 2001; Curtiss et al. 2002; Dahmann et al. 2011). Therefore, selector genes are responsible for attributing a unique property to the cells within their expression/functional domains. In the wing imaginal disc, *engrailed* (*en*) is expressed in the posterior compartment, and *apterous* (*ap*) is expressed in the dorsal compartment (Brower 1986; Cohen et al. 1992; Held 2002b; Hidalgo 1998), which serve as the selector for the posterior and dorsal fate, respectively (Table 1). The boundary between the cell populations of two compartments is the site for initiation of the signaling center, which regulates patterning, growth, and differentiation of the developing field (Blair 2001; Meinhardt 1983). Activation of the signaling centers at these developmental boundaries is responsible in maintaining the downstream patterning events (Blair 2001; Curtiss et al. 2002; Dahmann et al. 2011; Singh et al. 2012). This leads to an important question: How are these boundaries generated and maintained during development of a patterning field or an organ? In this chapter, we will provide an overview of recent advances on the genetic circuitry involved in generation of the boundary between the dorsal and ventral compartments, and its significance on development of an organ using *Drosophila* eye model. In this chapter, we will focus on the role of axial patterning genes in *Drosophila* eye development.

Table 1 Genes involved in axial patterning in developing imaginal discs of *Drosophila melanogaster*

Imaginal disc	Axis	Time	Selector genes	References
Wing	AP	L1	Anterior: *cubitus interruptus* Posterior: *engrailed, invected*	Lawrence and Morata (1976), Morata and Lawrence (1975), Sanicola et al. (1995)
	DV	L2	Dorsal: *apterous, Capricious, tartan, fringe, Serrate* Ventral: *Delta*, *wingless*	Blair et al. (1994), Cohen et al. (1992, 1993), Cohen (1993), Diaz-Benjumea and Cohen (1993)
	PD	L3	Proximal: *homothorax*, *teashirt* Distal: *nubbin, elbow, no ocelli*	Blair et al. (1994), Cohen et al. (1992, 1993), Diaz-Benjumea and Cohen (1993), Zirin and Mann (2007)
Leg	AP	L1	Anterior: *cubitus interruptus* Posterior: *engrailed, invected*	Dominguez et al. (1996), Eaton and Kornberg (1990), Kornberg et al. (1985), Masucci et al. (1990), Raftery et al. (1991), Zecca et al. (1995)
	DV	L2	Dorsal: *decapentaplegic* Ventral: *wingless*	Baker (1988b), Couso et al. (1993), Irvine and Vogt (1997), Zirin and Mann (2007)
	PD	L3	Proximal: *teashirt, homothorax* Distal: *distalless*	Diaz-Benjumea et al. (1994), Irvine and Vogt (1997), Lecuit et al. (1996)
Eye	DV	L2	Ventral: *Lobe* and *Serrate, Sloppy-paired* Dorsal: *pannier, Iroquois Complex, wingless*	Maurel-Zaffran and Treisman (2000), Oros et al. (2010), Sato and Tomlinson (2007), Singh and Choi (2003)
	AP	L3	Anterior: *eyeless* Posterior: *hedgehog*	Dominguez and Casares (2005), Halder et al. (1995), Lee and Treisman (2001)
	PD	L3	Proximo-Distal: Not fully understood	

AP antero-posterior, *DV* dorso-ventral, *PD* proximo-distal

Drosophila Eye Model to Study Axial Patterning

The power of *Drosophila* as a model organism for patterning and disease lies in its large repertoire of genetic tools available, making it a highly tractable model organism (Bier 2005; Singh and Irvine 2012; Tare et al. 2013a). The *Drosophila* eye has been extensively used (1) to investigate tissue patterning, growth, cell–cell communication, cell survival, and cell death mechanisms during organogenesis, and (2) to understand the genetic mechanism responsible for positional fate restrictions within a developing field that leads to formation of compartments (Dominguez and Casares 2005; Singh and Irvine 2012; Singh et al. 2005b, 2012). Interestingly, the eye as an organ has evolved independently as many as forty different times (Land and Fernald 1992). Despite the differences in the structure of *Drosophila's* compound eye and a vertebrate eye of a single lens and a retina with multiple layers of neurons, there is

similarity in the underlying genetic pathways controlling eye fate specification and differentiation. Thus, the genetic machinery involved in eye development is highly conserved and exhibits structural and functional similarity between insects and humans (Erclik et al. 2009; Gehring 2005; Hartenstein and Reh 2002; Kumar 2009; Wawersik and Maas 2000). This suggests that information generated in the fly eye can be extrapolated to the higher organisms. Therefore, *Drosophila* has proved to be an excellent model system for identifying new genes that are conserved in vertebrate retinal development (Singh et al. 2012).

Embryonic Eye Primordium Develops into the Larval Eye Disc in *Drosophila*

Drosophila, a dipteran, is a holometabolous insect (Anderson 1972b; Miall and Hammond 1892) where the primordia for all adult structures are first specified during embryonic development. The embryonic precursors grow asynchronously from the rest of the developing embryo (Anderson 1972a, b; Cohen et al. 1993; Cohen 1993; Crick and Lawrence 1975; Held 2002b; Kumar 2011; Singh et al. 2012). These embryonic primordia grow inside the larva as epidermal invaginations called imaginal discs (Atkins and Mardon 2009; Bodentstein 1950; Ferris 1950; Held 2002b). The *Drosophila* embryonic eye primordium originates from five embryonic head segments and the acron (Jurgens and Hartenstein 1993; Younossi-Hartenstein and Hartenstein 1993) and is specified by expression of *twin of eyeless (toy)* and *eyeless* (*ey*), a *Drosophila* homolog of human PAX6 (Quiring et al. 1994). The embryonic eye primordium begins as an antero-dorsal sac comprising of approximately 20 cells that are set aside during mid-embryogenesis (Garcia-Bellido and Merriam 1969; Held 2002b; Poulson 1950; Tsachaki and Sprecher 2012; Yamamoto 1996). These eye primordial cells continue to grow in the larva.

During larval development, the embryonic eye primordium develops into a monolayer epithelium, called the eye-antennal imaginal disc (Fig. 1a). 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 comprising of a columnar epithelium called the *d*isc *p*roper (DP) and a squamous epithelium called *p*eripodial *m*embrane (PM) (Atkins and Mardon 2009; Cho et al. 2000; McClure and Schubiger 2005). Fate map studies have revealed that the DP of the eye-antennal disc gives rise to the retina, whereas the PM forms the adult head structures (Atkins and Mardon 2009; Haynie and Bryant 1986; Milner et al. 1983; Singh et al. 2012). Earlier it was postulated that the PM is required during metamorphosis events of eversion and fusion. However, recent findings suggest that the PM is involved in sending signals to the DP and is required for cell survival and proliferation in the DP (Atkins and Mardon 2009). The eye-antennal imaginal disc upon differentiation gives rise to the adult eye, antenna, head cuticle, and other head

structures (Cohen 1993; Held 2002b). In the second instar larva, the division of the complex eye-antennal disc into the eye and antennal field occurs due to restriction of developmental potentials. This division occurs due to activation of the genetic circuitry required to initiate specification followed by differentiation of the eye and antenna (Atkins and Mardon 2009; Dominguez and Casares 2005; Kenyon et al. 2003; Kumar and Moses 2001). The developing eye field further gives rise to the eye proper, the head cuticle, and the ocelli, whereas the antennal field develops into the antenna and the head cuticle (Haynie and Bryant 1986).

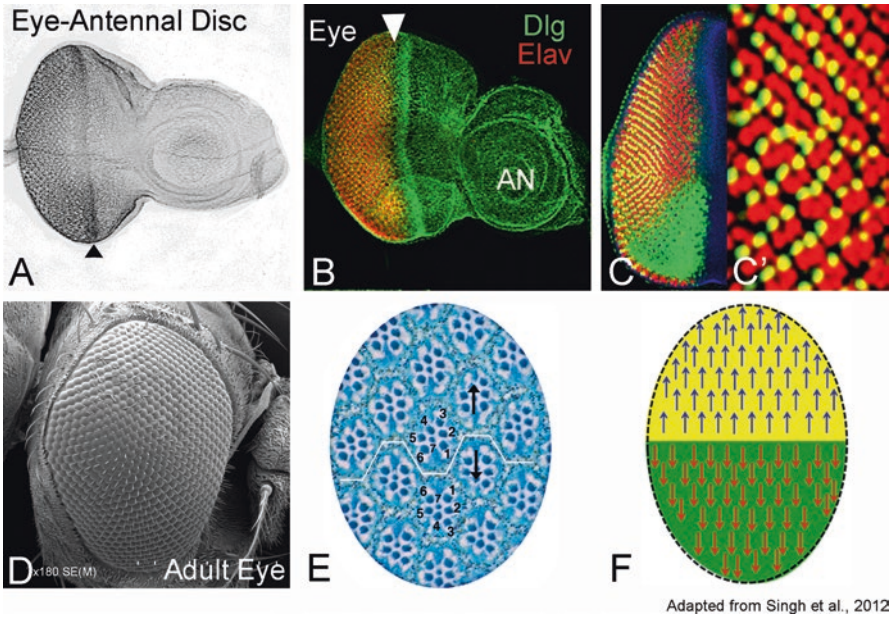

Fig. 1 DV patterning of the *Drosophila* compound eye begins in larval eye imaginal disc. (**a**–**c**) Eye antennal imaginal disc of a third instar larva. (**a**) Eye imaginal disc. (**b**) Eye antennal imaginal disc stained for membrane-specific marker Disc large (Dlg, green), and pan-neural marker Elav (red). Elav marks the photoreceptor neurons in the eye. Arrowhead in **a** and **b** marks the position of the morphogenetic furrow. (**c**, **c**′) Photoreceptor neurons exhibit DV polarity in the eye imaginal disc indicated by staining of Bar (**b**) antibody (green). (**d**) Scanning electron micrograph (SEM) of a wild-type adult *Drosophila* eye. The adult compound eye of *Drosophila* is made up of 750–800 unit eyes, each referred to as an ommatidium (Ready et al. 1976). All ommatidia are arranged in mirror image symmetry along the dorsal-ventral (DV) axis. (**e**) Each ommatidium consists of eight rhabdomeres which are organized as an asymmetric hexagonal structure. The DV polarity is determined by two different orientations of the ommatidia based on the orientation of R3 rhabdomere. R3 pointing upwards represents a dorsal ommatidium whereas R3 pointing downwards represents a ventral ommatidium. White line marks the equator. (**f**) Cartoon representing the mirror image symmetry of the ommatidia along the DV margins in the adult eye. Blue arrows in the yellow region mark the dorsal ommatidia, whereas red arrows in the green background mark the ventral ommatidia. Orientation of all images is dorsal up, ventral down, anterior right, and posterior left. *AN* Antenna

Like other dipteran insects, *Drosophila* has compound eyes for vision (Fig. 1d). The compound eye of the adult fly develops from the larval eye imaginal disc (Garcia-Bellido and Merriam 1969; Haynie and Bryant 1986; Peters 2002; Poulson 1950). The growth spurt occurs during early larval (first and second instar) eye development. During this stage, the undifferentiated cells of the eye-antennal imaginal disc cells divide and undergo rapid proliferation. During late second- or early third-larval instar stage, a synchronous wave of retinal differentiation is initiated in the eye imaginal disc. This indentation corresponds to the wave of retinal differentiation which initiates on the posterior margin of the eye disc and moves anteriorly and is referred to as the *m*orphogenetic *f*urrow (MF, Fig. 1a, b, arrowhead). This progressive pattern of MF results in the transition of an undifferentiated epithelium of retinal precursor cells to differentiated cell types comprising of regularly spaced photoreceptor clusters (Kumar 2013; Ready et al. 1976; Wolff and Ready 1993). The differentiating cells undergo an apical constriction and apico-basal contraction which results in an indentation in the eye imaginal disc. The photoreceptor clusters are generated posterior to the furrow 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 (Kumar 2011; Wolff and Ready 1993). Each photoreceptor neuron represents a unit eye, referred to as an ommatidium. The compound eyes in the adult fly consist of nearly 800 unit eyes called ommatidia (Fig. 1d). Each ommatidium is made up of approximately twenty cells. Of these, eight are distinct photoreceptor neurons (Fig. 1e) that project axons to the optic lobe of the brain. The remaining non-neuronal cells in an ommatidium are pigment cells, cone cells, and mechano-sensory bristles (Fig. 1d, e) (Held 2002b; Kumar 2011; Roignant and Treisman 2009; Singh et al. 2012; Wolff and Ready 1993).

The photoreceptor neurons are arranged in an asymmetric trapezoid or a hexagonal facet (Ready et al. 1976; Wolff and Ready 1993). The ommatidia within a compound eye are polarized in opposite directions. The orientation of one group of ommatidia is the mirror image of the other group (Fig. 1e, f). Furthermore, their orientation serves as a marker to distinguish the dorsal and ventral compartment specific fate in the larval eye imaginal disc (Fig. 1c, c′) as well as the adult compound eye (Fig. 1d–f). In the adult eye, the ommatidia possess mirror image symmetry along the dorso-ventral (DV) axis where these domains are referred to as the dorsal (D) and ventral (V) compartments. The border between these D and V compartments is referred to as an equator. The photoreceptor differentiation initiates on the posterior margin at the intersection of the D-V midline of the eye imaginal disc (Lee and Treisman 2002; Moses 2002). The delineation of DV midline or equator is responsible for signaling, which is crucial for photoreceptor development and differentiation. Thus, dorso-ventral (DV) patterning is an important facet of axial patterning during organogenesis. The DV polarity has been attributed to play a role in targeting of the retinal axons to the brain. This ommatidial configuration along with the targeting of the axons from the retina to the brain is a masterpiece of microptics and microcircuitry and enhances visual acuity and thereby forms the equator a sensitive “fovea” (Held 2002b). Interestingly, the eye imaginal disc is largely undifferentiated until

second instar of development. It raises an interesting issue of how and when the compartments are established in the *Drosophila* eye imaginal disc.

Sequence of Events During Axis Determination

The sequence of events during axial patterning of the wing and the leg imaginal discs involves first the division of a field into anterior and posterior compartments of independent cell lineages, which is defined by selector genes (Table 1). Generation of AP lineage is followed by subdivision of the wing and leg imaginal discs into dorsal and ventral compartments (Blair 2001; Singh et al. 2012). However, during eye imaginal disc development this sequence of division is not followed. The DV lineage is the first lineage restriction event in the developing eye (Oros et al. 2010; Singh and Choi 2003; Singh et al. 2005b, 2012, 2019; Tare et al. 2013a). The antero-posterior axis, which follows later, is generated with the onset of differentiation marked by the MF in early third instar of larval development. The MF sweeps across the eye imaginal disc from the posterior margin towards anterior resulting in the formation of posterior fate behind the furrow (Kumar 2011; Ready et al. 1976; Wolff and Ready 1993). In majority of insects, including *Drosophila*, there is no well-defined PD axis as the adult eye is present in a socket in the head (Singh et al. 2019). The entire early eye primordium is ventral in fate and on which the dorsal fate is established in early second instar of larval eye development (Singh and Choi 2003; Singh et al. 2005b). Therefore, DV patterning, which is established as early as early second instar of eye development, is the first lineage restriction in the eye imaginal disc (Singh and Choi 2003; Singh et al. 2005b, 2012). Even though there are differences in the sequence of events, evidences suggest that some aspects of the DV patterning mechanism are highly conserved in the developing eye and the wing. One of the common features among all these organ primordia is the generation of the DV boundary, which serves as the site for activation of the signaling pathways to trigger growth and patterning of the imaginal disc (Tare et al. 2013a). Here our emphasis will be on the mechanism of generation of DV domains, and how it regulates growth and patterning in the developing eye.

Generation of Dorsal and Ventral Compartments in the Developing Eye Disc

The *Drosophila* eye is a polarized tissue. The polarity in *Drosophila* eye is reflected by mirror image arrangement of ommatidia across the DV midline or equator (Fig. 1f). The relation between the equator and DV compartmental boundary has been a matter of debate for a long time. The equator was first reported by Wilhelm Dietrich (1909). In many insect eyes, the equator has been described as the boundary

between the photoreceptor neurons of the dorsal and ventral compartments (Dietrich 1909). The equator is generated upon specification of dorsal and ventral compartments and serves as the signaling center, which is crucial for cell proliferation and differentiation of the eye as an organ. The *Drosophila* eye model has been extensively used to unravel the molecular genetic mechanisms underlying this crucial process of generation of DV compartments in the eye (Singh et al. 2005b, 2012; Tare et al. 2013a). Since the developmental mechanisms underlying the DV pattern are not fully understood, it raises an interesting question of how the dorsal and ventral pattern is established in the developing eye.

Earlier studies employed the genetic mosaic approach to study DV pattern generation in the developing eye. Hans Becker reported that clones respect the equator and do not cross the DV lineage boundary (Becker 1966; Held 2002b). The pioneering studies authored by Donald Ready, Thomas Hansen, and Seymour Benzer (1976) entitled "Development of the *Drosophila* retina, a neurocrystalline lattice" provided insights into patterning in the *Drosophila* eye (Ready et al. 1976). They rejected the clonal analysis model of ommatidial lineage (Kankel et al. 1980). They employed a genetic mosaic approach to generate mitotic recombination between the *white*$^{+}$ *(w*$^{+}$*)* wild-type and w^{-} mutant chromosomes. Their aim was to generate two new cell populations w^{-}/w^{-} and w^{+}/w^{+} clones in a w^{+}/w^{-} paternal heterozygous background. The w^{+} gene is essential for red eye pigment uptake in the cells and serves as an excellent cell-autonomous marker for photoreceptors and pigment cells (Lawrence and Green 1979; Ready et al. 1976). They found that in genetic mosaic w^{-} clones generated in the dorsal half of the eye can cross a few cells into the ventral half and *vice versa*. The results from these studies in the *Drosophila* eye suggested that the equator is not determined as the boundary between the D and V cell lineages (Ready et al. 1976). Although, 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 (Netter et al. 1998).

In a series of elegant genetic analysis experiments involving a large number of mosaic clones in the adult eye, Baker (1978) demonstrated that clones strictly follow the DV boundary, and do not intermingle near the DV border (Held 2002b; Singh et al. 2012). These studies validated the hypothesis that the *Drosophila* eye is derived from dorsal and ventral compartments. To analyze whether the eye and the head are also subdivided into different domains by sequential compartmentalization, a mosaic analysis was carried out. Nearly all clones (96%) respected the DV boundary (do not cross the boundary) and were restricted to either dorsal or ventral domain of the eye. 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 formation of dorsal and ventral compartment boundary. Alternatively, 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; Singh et al. 2005b, 2012; Tare et al. 2013a). 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 in the larval eye imaginal disc. These clones showed a sharp outline

along the DV midline and the clones located within the dorsal or ventral domain had wiggly borders (Dominguez and de Celis 1998). Later, it was established that DV lineage specification is the first event that occurs during organogenesis of the eye (Singh et al. 2012). Therefore, studying the major developmental landmarks along the temporal axis is important to understand the process of patterning and growth of this organ.

Genesis of the Eye

Activation of Notch (N) signaling at equator, the boundary between dorsal and ventral compartments, has been shown to promote growth, in establishing planar polarity, in spacing of ommatidial clusters, and in cell fate specification and differentiation (Baonza and Garcia-Bellido 2000; Cagan and Ready 1989; de Celis et al. 1996; Go et al. 1998; Singh et al. 2012). However, this argument of DV patterning being crucial for growth does not suitably fit the time line of developmental events (Singh et al. 2012; Tare et al. 2013a). If ommatidial orientation corresponds to the generation of the DV axis then based on the time point when the ommatidial rotation occurs (Mlodzik 1999; Reifegerste and Moses 1999), the majority of the growth and cell proliferation of the developing eye field is already accomplished. The ommatidial orientation of the photoreceptors occurs in the pupal retina and growth spurt occurs during early larval instars of eye imaginal disc development. Based on the earlier notion, if DV patterning occurs in the pupal retina, then its role in growth and differentiation cannot be explained as both growth and differentiation occur prior to it during imaginal disc development, and not in the adult eye. Furthermore, the major growth spurt in imaginal disc development occurs during larval stages. Thus, efforts were channeled towards investigating the timeline and the genetic control that initiates DV patterning during eye development. Therefore, efforts were directed to (a) understand the time point of generation of DV axis in the developing eye or (b) identify the developmental event which corresponds to the onset of N signaling in the developing eye (Singh et al. 2012; Tare et al. 2013a).

Three different groups provided evidences in their independent publications that DV lineage restriction takes place earlier in larval eye imaginal disc due to domain-specific expression of the genes. These genes are referred to as the DV patterning genes (Cho and Choi 1998; Dominguez and de Celis 1998; Papayannopoulos et al. 1998). These genes may be involved in assigning, generating, and maintaining the DV lineage in the developing eye imaginal disc. A new time line assigned the time window of initiation of DV patterning to early larval eye development. This hypothesis also fits with the logic of a growth spurt. They identified the domain-specific expression of these genes whose function also follows the DV domain constraint is established during early larval stages of development (Cho et al. 2000; Cho and Choi 1998; Dominguez and de Celis 1998; Papayannopoulos et al. 1998; Singh et al. 2012; Tare et al. 2013a).

These studies raised a new question: if DV patterning occurs so early in the developing eye disc, then what is the default state of 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. Several studies have reported the genes that are expressed in the early larval eye primordium. It is known that the generation of MF marks the formation of AP axis in early third instar of larval eye imaginal disc development (Kumar 2013; Ready et al. 1976; Wolff and Ready 1993). However, the DV axis is determined as early as the late first instar of larval development by domain-specific expression of genes along the DV axis (Cho and Choi 1998; Dominguez and de Celis 1998; Papayannopoulos et al. 1998; Singh and Choi 2003; Singh et al. 2012). Another interesting outcome from the Singh and Choi (2003) studies was that early eye primordium begins from a default ventral state (Fig. 2), which depends on the function of ventral genes like *Lobe (L)* and its downstream target *Serrate (Ser)* (Kumar 2011; Singh et al. 2005a, b; Singh and Choi 2003). It has been shown that loss-of-function of *L/Ser* results in preferential loss of ventral eye (Figs. 2, 3c, d). L is expressed uniformly in the entire eye imaginal disc (Figs. 2, 3a). 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, 2012). During early eye development, the loss-of-function of *L* results in the complete loss of the eye field (Figs. 2, 3c, d). However, loss of the *L* gene function later during eye development causes selective loss of the ventral half of the eye (Fig. 2, Singh et al. 2012). Loss-of-function of *Ser* also results in the similar loss of ventral eye phenotype (Kumar and Moses 2001; Singh and Choi 2003; Singh et al. 2005b, 2012). Interestingly, the timing of restriction of the *L/Ser* functional domain from the entire developing eye field (Fig. 3e, f) to only the ventral half of eye (Fig. 3c, d) corresponds to the onset of GATA-1 transcription factor encoding *pannier (pnr)* gene expression along the dorsal margin of the eye (Table 2; Fig. 2). During late first instar larval eye development, the entire homogenous population of the ventral cells of the eye primordium transitions into two distinct dorsal and ventral lineages with the onset of *pnr* expression on the dorsal eye margin (Oros et al. 2010; Singh and Choi 2003; Singh et al. 2012). 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). In the subsequent parts of this chapter, we will focus on specific functions of DV patterning genes responsible for pattern generation in the developing eye.

DV Patterning During Imaginal Disc Development

The DV axis is determined by domain-specific expression or function of DV patterning genes (Singh et al. 2012; Tare et al. 2013a). However, their localization may not be identical in all the imaginal discs. Unlike the wing imaginal disc where *Ser* and *Delta (Dl)* are preferentially expressed in the dorsal and ventral domains,

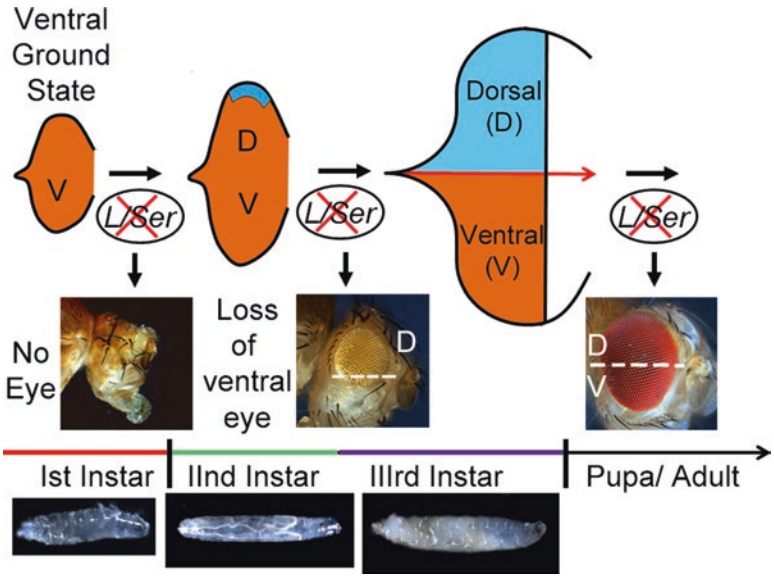

Fig. 2 Ventral is the default state of/in the developing *Drosophila* eye. Larval eye primordium begins with a default ventral state where all the cells of the eye primordium require ventral genes *L/Ser* function for growth and proliferation (Singh and Choi 2003; Singh et al. 2012). Loss-of-function phenotype of *L/Ser* in the developing eye imaginal disc evolves progressively 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. During early second instar of larval eye development, a few cells start expressing *pnr* and the dorsal boundary is specified. By the end of the second instar stage, DV lineage is established and at this stage, loss of *L/Ser* results in loss of only the ventral half of the eye. In the late third instar stage of development when retinal differentiation is complete, loss of *L/Ser* does not have significant effect on the overall adult eye morphology. These results clearly indicate that the entire early eye primordium, prior to onset of *pnr* expression, is ventral in fate (Singh and Choi 2003). *DV* dorso-ventral

respectively, their expression domains are reversed in the eye imaginal disc (Table 1). In the wing imaginal disc, the LIM homeodomain protein Apterous (Ap) acts as a dorsal fate selector (Table 1) (Blair et al. 1994; Cohen et al. 1992). It is known that Ap can induce Fringe (Fng) and Ser in the dorsal compartment of wing imaginal disc (Bachmann and Knust 1998; Cohen et al. 1992; de Celis et al. 1996; Diaz-Benjumea and Cohen 1995). The distribution of N ligands in the eye disc is reversed when compared to the wing imaginal disc as Ser expression is restricted to the dorsal compartment of the wing, whereas Dl expression is observed in the ventral compartment of the wing imaginal disc. In the eye imaginal disc, Dl and Ser are preferentially expressed in the dorsal and ventral domains, respectively (Cho et al. 2000; Cho and Choi 1998; Struhl 1981). Therefore, in the wing imaginal disc, Ser functions as a N ligand in the dorsal cells, whereas Dl is the N ligand in the ventral cells. Furthermore, *fng* is ventral-specific in the eye (Cho and Choi 1998) but dorsal-specific in the wing imaginal disc (Singh et al. 2012). This mirror image reversal in the distribution of the dorsal and ventral genes in the eye versus wing imaginal disc

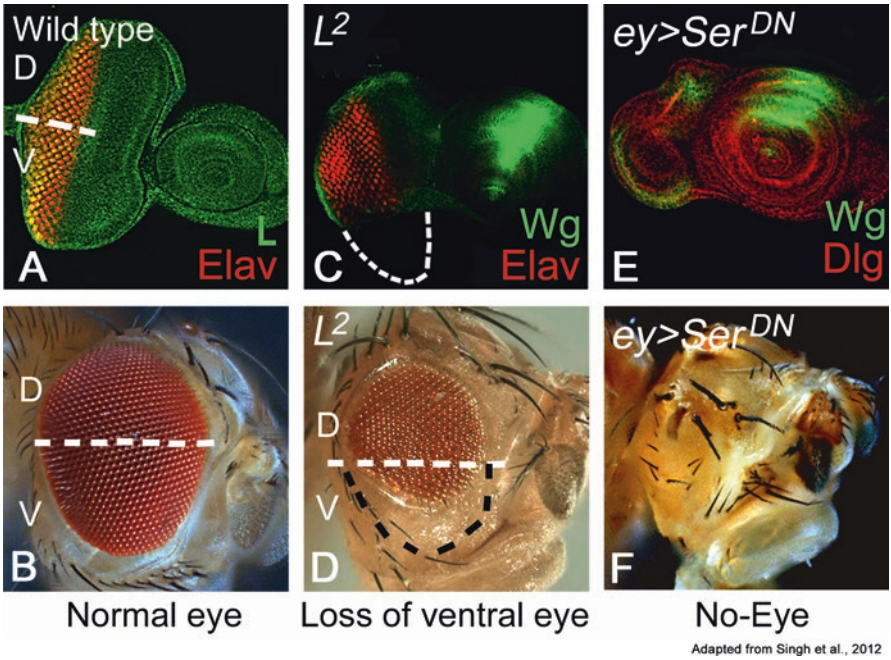

Fig. 3 *Lobe (L)* and *Serrate (Ser)* are required to promote cell survival in the developing eye imaginal disc (Singh et al. 2006). (**a**) In the wild-type eye imaginal disc, L (green) is expressed ubiquitously throughout the eye disc and Elav (red) marks the photoreceptor neurons. (**b**) Wild-type adult eye. White dotted line separates dorsal (D) half of the eye from ventral (V). (**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 compartments. 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 disc using an *ey*-Gal4 driver results in complete loss of eye field both in (**e**) the early eye imaginal disc, and (**e**) the adult

is probably due to the fact that the eye disc rotates 180° during embryogenesis and as a result, the DV axis is inverted in the eye with respect to the wing disc (Struhl 1981). Despite the differences in distribution, similar to the developing eye imaginal disc, Fng is still required for N activation at the DV border in the wing imaginal disc. Fng, a glycosyltransferase, elongates O-linked fucose residues to EGF domains of N to promote N-Dl interaction and thereby modulate N signaling (Okajima and Irvine 2002). Contrary to it, Fng inhibits Ser-N interaction (Ju et al. 2000; Moloney et al. 2000). The genes evolved in DV patterning can be categorized in three broad categories:

Table 2 Genes involved in dorso-ventral (DV) patterning and domain-specific expression and growth

Drosophila	Vertebrate homolog	Nature	Function in eye	References
Ventral genes				
Serrate (Ser)	Jagged-1	N ligand in the ventral eye	Growth and development of ventral eye	Cho et al. (2000), Cho and Choi (1998), Dominguez and de Celis (1998), Papayannopoulos et al. (1998), Speicher et al. (1994)
Lobe (L)		Proline rich Akt substrate	Ventral eye growth and survival, has no effect on dorsal eye growth	Chern and Choi (2002), Singh et al. (2005a, b, 2006), Singh and Choi (2003), Wang and Huang (2009)
Fringe (fng)	Lunatic fringe	Glycosyl transferase	Secreted signaling protein, DV boundary formation	Cho and Choi (1998), Dominguez and de Celis (1998), Irvine and Wieschaus (1994), Papayannopoulos et al. (1998)
Chip (Chi)	Nli/Ldb1/ Clim-2	Ubiquitin Ligase, Transcription co-factor	Define ventral eye boundary	Roignant et al. (2010)
sloppy-paired (slp2)	BF-1 (not complete homology)	Forkhead transcription factor	Ventral eye growth	Sato and Tomlinson (2007)
decapentaplegic (dpp)	BMP	TGF-β	Ventral growth	Chanut and Heberlein (1997a), Singh et al. (2005b)
Dorsal genes				
pannier (pnr)	GATA-4	Zinc finger, GATA family	Dorsal eye fate selector	Gomez-Skarmeta and Modolell (2002), Maurel-Zaffran and Treisman (2000), Oros et al. (2010), Ramain et al. (1993), Singh et al. (2005b)
araucan (ara)	Irx 1, 3	Homeodomain	Dorsal eye fate selector	Cavodeassi et al. (1999), Gomez-Skarmeta and Modolell (1996, 2002), Pichaud and Casares (2000)

(continued)

Table 2 (continued)

Drosophila	Vertebrate homolog	Nature	Function in eye	References
caupolican (caup)	Irx2, 5	Homeodomain	Dorsal eye fate selector	Cavodeassi et al. (1999), Gomez-Skarmeta and Modolell (1996, 2002), Pichaud and Casares (2000)
Delta (Dl)	Delta like 3 (DLL3)	Transmembrane Notch Ligand	Dorsal Notch (N) Ligand	Cho et al. (2000), Cho and Choi (1998), Dominguez and Casares (2005), Dominguez and de Celis (1998), Papayannopoulos et al. (1998), Singh et al. (2005b)
Asymmetrically expressed genes regulating domain-specific growth (i) Functional domain is restricted only to the ventral half of the eye				
homothorax (hth)	Meis	Homeodomain	Negative regulator of eye, ventral eye suppression	Bessa et al. (2002, 2008), Pai et al. (1998), Pichaud and Casares (2000), Singh et al. (2005b, 2011, 2012)
(ii) Functions differently in the dorsal and ventral half of the eye				
teashirt (tsh)	TSH1, TSH2, TSH3	C2H2 zinc finger transcription factor	DV asymmetric function, promote dorsal eye growth, ventral eye suppression	Bessa and Casares (2005), Bessa et al. (2002), Datta et al. (2009), Pan and Rubin (1998), Singh et al. (2002, 2004, 2005b, 2012)
(iii.a) Marginally expressed genes				
optomotor blind (omb)	Tbx5	Transcription factor	Cell proliferation	Calleja et al. (1996), Singh et al. (2004), Tare et al. (2013b)
wingless (Wg)	Wnt	Signaling/ Secreted Morphogen	Eye growth, negative regulator of eye differentiation, promote head fate	Heslip et al. (1997), Legent and Treisman (2008), Treisman and Rubin (1995)
dachsous (Ds)	DCHS1	Cell adhesion and molecule binding	Ds transcription is regulated by Wg that negatively regulates fj gradient. Regulation of planar cell polarity	Simon (2004), Singh and Mlodzik (2012), Willecke et al. (2008), Zeidler et al. (1999a)

(continued)

Table 2 (continued)

Drosophila	Vertebrate homolog	Nature	Function in eye	References
(iii. b) Equatorially expressed genes				
four-jointed (fj)	FJX1	Type II transmembrane protein/secreted protein, kinase activity	Proliferation, planar Cell polarity, regulate its own expression	Bosveld et al. (2012), Brodsky and Steller (1996), Zeidler et al. (1999a)
unpaired (upd) also known as outstretched (os)	Leptin family of pathway ligands	Secreted glycosylated protein	Posterior midline, acts downstream of *four jointed,* regulates eye size through the JAK/STAT signaling, regulate cell cycle and cell proliferation.	Bach et al. (2007), Langer et al. (2004), Tsai and Sun (2004)

Genes Regulating Ventral Eye Growth

Axial patterning marks the generation of AP, DV, and PD axes. Out of all these three axis, the generation of DV axis marks the first lineage restriction event in *Drosophila* eye. The domain-specific expression and function of DV patterning genes divide a developing eye field into dorsal and ventral compartments. The DV patterning genes have been classified into dorsal or ventral genes based on their domain-specific expression, function, or both (Table 2). The ventral eye genes include several genes like *fng*, *L*, *Ser, chip (Chi)*, and *sloppy-paired (slp)* (Table 2). Among these genes, *L* was first reported in 1925 as a gene required for eye growth (Morgan et al. 1925). Based on the *L* mutant phenotypes of preferential loss of the ventral half of the eye, it was suggested that *L* is required for growth and differentiation of ventral half of the eye (Chern and Choi 2002; Singh et al. 2005b, 2012). Genetic analysis demonstrated that ventral eye specific function of *L* was downstream to N signaling (Chern and Choi 2002). These results further validated the hypothesis that asymmetric function of DV patterning genes regulates growth in early eye disc.

The genetic epistasis analysis revealed that *L* acts upstream of *Ser*, a N ligand in the ventral eye (Chern and Choi 2002; Cho and Choi 1998; Dominguez and Casares 2005; Dominguez and de Celis 1998; Papayannopoulos et al. 1998; Speicher et al. 1994). Furthermore, *Ser* transcription (based on *Ser*-lacZ reporter expression) is repressed in the early eye discs from L^{si} homozygous larvae (Chern and Choi 2002). Loss-of-function clones of *L* in the eye imaginal disc resulted in strong downregulation of *Ser* in the ventral eye, whereas increased levels of *L* using the random "flp-out" approach induced *Ser* expression even in the dorsal domain of eye imaginal disc (Chern and Choi 2002). These studies assigned *L* to a genetic hierarchy of ventral eye genes (Table 2), where *L* acts downstream of N and acts upstream of *Ser* in the developing eye imaginal disc (Chern and Choi 2002). The reduced eye size

seen in the hypomorphic alleles of *Ser* further validated the role of *Ser* in early eye development. Surprisingly, loss-of-function clones of *Ser* in the eye did not result in a reduced-eye phenotype (Chern and Choi 2002; Papayannopoulos et al. 1998; Sun and Artavanis-Tsakonas 1996). However, misexpression of dominant negative form of *Ser* (Ser^{DN}) (Fleming et al. 1997) in the entire early eye imaginal disc using *ey*-Gal4 driver (Hazelett et al. 1998) results in either preferential loss of ventral eye or loss of the entire eye (Kumar and Moses 2001; Singh and Choi 2003; Singh et al. 2005b, 2012). Random gain-of-function clones of Ser^{DN} generated by the "flp-out" method (Pignoni and Zipursky 1997) also resulted in suppression of eye fate in the ventral eye. Lack of phenotype in *Ser* mutant clones can be attributed to compensation of *Ser* function by another factor. Alternatively, cell culture experiments suggested that Ser may secrete or transendocytose into neighboring cells (Klueg and Muskavitch 1999; Kumar and Moses 2001; Singh et al. 2005b, 2012). Similar phenotypes of Ser^{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 (Singh et al. 2012). In a forward gain-of-function genetic screen using EP lines, many downstream genetic modifiers of *L* dominant mutations were identified (Singh et al. 2005a). This screen resulted in identification of the role of *L* in cell survival and developmental cell death (Singh et al. 2006). These studies further shed light on how axial patterning genes utilize highly conserved Wingless/Wnt (Wg)signaling pathway and Jun-N-Terminal Kinase (JNK) signaling pathways to promote cell survival and growth.

Fng is known to bind N to promote N-Dl interaction, and is required to restrict N activation at the DV border (Fleming et al. 1997; Irvine and Wieschaus 1994; Kim et al. 1995). 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; Moloney et al. 2000; Singh et al. 2005b, 2012). As a result, the N activation by Dl is enhanced only at the DV border. The DV patterning genes expression pattern changes dynamically in the developing eye imaginal disc. Consequently, the striking differences exist in the expression patterns before and after the initiation of the retinal differentiation. For example, during early eye imaginal disc development, *fng* is expressed in the ventral domain, which is just opposite to the expression of the dorsal fate selector gene *mirror, (mirr)* (Papayannopoulos et al. 1998). However, as the eye imaginal disc undergoes retinal differentiation and the morphogenetic furrow (MF) proceeds anteriorly, *fng* exhibits preferential localization anterior to MF both in the dorsal and ventral eye domain (Cho and Choi 1998), and is thus known to regulate signaling between both dorsal and ventral cell interactions (Irvine and Wieschaus 1994). 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 loss-of-function clone of *fng* further emphasized the important role of Fng in DV patterning in the eye. Loss-of-function clones of *fng* in the ventral eye exhibit reorganization of DV polarity near the ectopic fng^{+}/fng^{-} border, which results in non-autonomous polarity reversals. It results in the generation of *de novo* equators and ectopic localized activation of N at the fng^{+}/fng^{-} boundary (Baonza and Garcia-Bellido 2000; Cho and Choi 1998; de Celis et al. 1996; Go et al. 1998).

These observations suggest that (1) Fng has an essential role in DV patterning and (2) the DV pattern is established prior to retinal differentiation during the early eye development.

Other ventral eye genes are *Chip* and *slp 1 or 2* (Table 2). Chip, an ubiquitin ligase, acts as a ubiquitous transcriptional co-factor. Chip interacts with classes of transcription factor during neural development and is known to establish the ventral boundary of the eye and the head tissue (Roignant et al. 2010). Loss-of-function of *Chip* has been shown to induce ectopic retinal differentiation in the ventral eye. Therefore, possible function of *Chip* is to prevent ectopic retinal differentiation at the ventral eye-antennal disc boundary and thereby promote the head-specific fate. The eye repression function of Chip is mediated *via* interactions with LIM homeodomain proteins: Arrowhead (Awh) and Lim 1. Chip and Lim1 repress the selector gene *eyeless (ey)* to prevent ectopic differentiation (Roignant et al. 2010). Thus, LIM-HD/Chip complex is required for defining the boundary between eye and head field. However, the eye suppression activity of Chip is independent of Meis class protein Hth or its co-factor Exd. Although, it is predicted that Chip and Hth act independently, but parallel to each other in order to suppress eye fate on the ventral eye margin (Roignant et al. 2010). Other ventral eye genes *slp1* and *slp2* encode homologous Forkhead transcription factors that are known to have redundant roles during embryonic patterning (Grossniklaus et al. 1992). In the developing eye, Slp proteins in the ventral eye repress and thereby restrict Iro-C proteins to the dorsal compartment. During early stages of development, Slp and Iro-C abut the DV border. N signaling activation at the equator results in downregulation of *slp* and a gap is generated between the expression domains of Slp and Iro-C, which is necessary for induction of N ligands Dl in the dorsal and Ser in the ventral cells. Thus, repressive interaction between *slp* and *N* promotes the emergence of Ser and Dl expressions in the eye (Sato and Tomlinson 2007).

A member of the TGF-β family, *decapentaplegic (dpp),* is another possible ventral eye gene. *dpp* is a homolog of transforming growth factor-β (Padgett et al. 1987; Spencer et al. 1982). It exhibits preferential expression in the ventral eye domain of the early eye imaginal disc (Cho et al. 2000; Won et al. 2015). During early eye development stages (before the progression of morphogenetic furrow), *dpp* (with *hh*) prevents dorsal fate by repressing the expression of both *wingless (wg)* and *orthodenticle (otd).* While during later stages, *dpp* (along with Hedgehog, Hh) plays crucial role in the progression of morphogenetic furrow (MF) progression from posterior end towards the anterior end in eye-antennal imaginal discs (as seen by failures in MF progression, in loss-of-function mutants of *dpp*) (Heberlein et al. 1993). Interestingly, the genetic tools/reagents for *dpp* expression like *dpp*-LacZ exhibit dynamic nature of *dpp* expression, which moves along with the morphogenetic furrow (MF). However, the *dpp*-Gal4 drivers, which are commonly available, do not follow this dynamic *dpp* expression that moves with the MF. Instead, majority of them drives expression along the posterior margin of the developing eye imaginal disc (Sarkar et al. 2018a). Recently, using GMR lines collection where regulatory regions of *dpp* are fused with Gal4 coding region, a MF-specific *dpp*-Gal4 driver was identified. Two new *dpp*-Gal4 lines which carry sequences from

first intron region of *dpp* gene were identified. GMR17E04-Gal4 drives expression along the MF during development and later in the entire pupal retina, whereas GMR18D08-Gal4 drives expression the entire developing eye disc, which later drives expression only in the ventral half of the pupal retina (Sarkar et al. 2018a).

During eye development, Dpp acts as a long range secreted morphogen (Chanut and Heberlein 1997b; Nellen et al. 1996). It is known to form a morphogen gradient in the early eye anlage (anterior brain and eye field) (Chang et al. 2001). Mutants of *dpp* exhibit similar pattern defects in the ventral eye disc as seen in *L* mutants. This *dpp* mutant phenotype may be an outcome of ectopic induction of dorsal eye genes *pnr, iro-C members* or *wingless* (*wg*) expression in the ventral domain as observed in *L* mutants (Singh et al. 2005a). Dpp, Hedgehog (Hh), and Wg signaling from the PM is required to trigger N activation in the DP of the early eye imaginal disc. During eye imaginal disc development, Dpp antagonizes Wg in the eye-antennal imaginal discs. This antagonistic interaction between *dpp* and *wg* divides the anterior compartment of the eye discs into two halves (dorsal and ventral), thereby creating a dorso-ventral (DV) axis that helps maintaining chirality in the developing structures (Theisen et al. 1996). This developmental interaction between Wg and Dpp in the eye is similar to that observed during limb development (Brook and Cohen 1996; Penton and Hoffmann 1996; Theisen et al. 1996). This antagonistic interaction occurs in the PM across the DV border (Cho et al. 2000). Thus, Dpp signaling plays a role in inducing DV polarity.

Dorsal Fate Selector Genes

The compartment boundaries are defined by the spatio-temporal expression or function of the fate selector genes. Loss-of-function of these selector genes results in the loss/elimination of that particular fate in the developing field (Blair 2001; Curtiss et al. 2002; Dahmann et al. 2011). In the *Drosophila* eye, these selector genes were identified in the earlier enhancer trap screens (Bhojwani et al. 1995; Bier et al. 1989; Singh 1995; Singh et al. 2012; Sun et al. 1995). These enhancer trap lines had *mini-white* (*w*) and *lacZ* reporter gene (*P-lac*W) (Bellen et al. 1989; Bhojwani et al. 1995; Bier et al. 1989; Singh 1995; Sun et al. 1995; Wilson et al. 1989), which exhibited domain-specific expression in the developing as well as the adult eye. These enhancer trap lines have made significant contributions towards understanding the DV patterning in the eye (Choi et al. 1996; Kehl et al. 1998; McNeill et al. 1997; Morrison and Halder 2010; Sun and Artavanis-Tsakonas 1996). Some of the lines identified had w^+ expression restricted only to the dorsal half of the adult eye. Most of these dorsal-specific P-element insertion lines were mapped to the chromosomal region 69CD. The molecular characterization of this 69CD chromosomal region, which was identified as a hot spot for P-*lac*W insertions, revealed the existence of a homeoprotein (homeobox genes), transcription factors from TALE class (Bürglin 1997). These transcription factors are encoded by Iroquois (Iro-C) gene complex, which comprise of three genes, *araucan* (*ara*), *caupolican* (*caup*), and *mirror*

(*mirr)*. These *Iro-C* genes are specifically expressed in the dorsal half of the *Drosophila* eye (Table 2) (Fig. 7b, b') (Gomez-Skarmeta et al. 1996; Grillenzoni et al. 1998; Heberlein et al. 1998; Kehl et al. 1998; McNeill et al. 1997; Singh et al. 2005b). The genomic organization, arrangement of these three genes as a cluster remains conserved from flies to mammals (Cavodeassi et al. 2001; Gómez-Skarmeta and Modolell 2002). This cluster of homeobox genes, *araucan* (*ara*), *caupolican* (*caup*), and *mirror* (*mirr*) (Table 2) (Gomez-Skarmeta and Modolell 1996; Grillenzoni et al. 1998; Heberlein et al. 1998; Kehl et al. 1998; McNeill et al. 1997; Singh et al. 2005b) are located within an approximately 140Kb region (Netter et al. 1998), and are expressed in the dorsal half of the eye (Fig. 7b, b′). They are referred to as Iroquois complex (Iro-C) as the mutation in these genes results in lack of lateral thoracic bristles in *Drosophila,* which resembles the hair style of the Indian tribe, the Iroquois (a native tribe which shaved all but a medial stripe of hair on the head and are also called Mohawks) (Gomez-Skarmeta and Modolell 1996; Leyns et al. 1996). They named the genes Araucan and Caupolican in honor of an Amerindian tribes, Araucanians, and one of their heroes—Caupolican. The third member of this complex was named *mirror (mirr)*. Together this complex is known as Iroquois complex (Gómez-Skarmeta and Modolell 1996; Leyns et al. 1996). Mirror is expressed in central nervous system (Netter et al. 1998; Urbach and Technau 2003) and is also involved in follicle cell patterning (Jordan et al. 2000), while *ara* and *caup* are expressed in mesodermal tissues in embryos. The members of Iro-C are highly conserved essential genes and exhibit significant differences in their expression pattern (Gomez-Skarmeta and Modolell 2002). However, there is functional redundancy in terms of Iro-C members between flies and higher vertebrates. Mirr is strongly and dynamically expressed in the central nervous system (Netter et al. 1998; Urbach and Technau 2003) and is essential for follicle cell patterning (Jordan et al. 2000). The other members Ara and Caup are preferentially expressed in mesodermal tissues in the embryos (Netter et al. 1998). The expression of all three Iro-C members is restricted to the dorsal half of the eye imaginal discs (Fig. 4e, 7b, b′), raising a possibility that they might be functionally redundant. Loss-of-function of *mirr* using $mirr^{e48}$ allele showed 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). The cells from two different compartments are of different cell lineages and do not intermingle due to differences in cell identities and affinities (Dahmann et al. 2011; Garcia-Bellido et al. 1973; Irvine 1999). Loss-of-function clones of *mirr* in the dorsal half of eye exhibit smooth clone borders, whereas in the ventral half of the eye shows wiggly clone borders (Yang et al. 1999). It suggests that dorsal eye cells lacking *mirr* avoid mixing with the neighboring *mirr* expressing cells. Furthermore, the dorsal clones exhibit dorsal eye enlargements and the polarity of the ommatidia in *mirr* loss-of-function clones is reversed. This analysis suggests that *mirr* functions as a dorsal fate selector. 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 was resolved when a deficiency iro^{DMF3} which uncovers all three *Iro-C* genes by the deletion of *ara* and

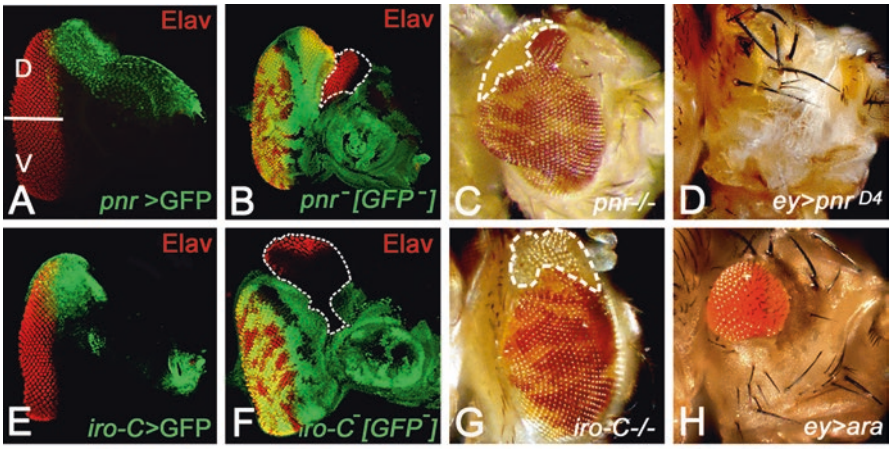

Fig. 4 Pnr and Iro-C members function as dorsal eye fate selectors. (**a**) Pnr expression (green) is restricted to the dorsal eye margin of the developing eye imaginal disc. Elav (red) marks the photoreceptor neurons. (**b**, **c**) Loss-of-function clones of *pnr,* marked by absence of GFP reporter (Green), results in the enlargement of 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 anterior region of the eye imaginal disc, which is attributed by generation of *de novo* equator in the dorsal compartment of eye imaginal disc. (**d**) Misexpression of *pnr* (*ey* > pnr^{D4}) in the eye imaginal disc suppresses the eye fate validating a late function of *pnr* in defining the eye field boundary (Maurel-Zaffran and Treisman 2000; 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 eye imaginal disc (*ey* > *ara*) results in a small eye. *D* Dorsal, *V* Ventral

caup as well as a 5′-region of *mirr* was employed for clonal analysis (Diez del Corral et al. 1999; Gomez-Skarmeta et al. 1996; Gomez-Skarmeta and Modolell 1996). 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 (Fig. 4f, g). There was no phenotype in the ventral half of the eye. Gain-of-function of Iro-C members in the eye results in reduced-eye phenotype (Fig. 4h). These results further highlighted the importance of the boundary between the dorsal and ventral cell types. These results strongly support that the three members of *Iro-C* 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 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 the *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, *pannier (pnr) is* another dorsal gene, expressed in the dorsal eye margin (Fig. 4a; 7a, a′). Pnr has two zinc finger motifs. Pnr was initially identified in EMS screen where *pnr* lethal mutation exhibits abnormalities with larval cuticle (Jürgens et al. 1984). During eye development, *pnr* exhibits similar loss-of-function (Table 2; 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; Oros et al. 2010; Pichaud and Casares 2000; Singh et al. 2005b). 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 (Dominguez and Casares 2005; Maurel-Zaffran and Treisman 2000; Oros et al. 2010; Pichaud and Casares 2000; Ramain et al. 1993; Singh et al. 2005b). In the hierarchy of dorsal genes, *pnr* is the topmost gene, and induces Wg which in turn induce the expression of downstream target genes *mirr* in the dorsal half of the eye (Dominguez and Casares 2005; Maurel-Zaffran and Treisman 2000; 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).

Our lab identified *defective proventriculus* (*dve*) as a new member of dorsal eye gene hierarchy (Puli and Singh et al. submitted). *dve* is a K50 homeodomain protein, named after defective morphogenesis of proventriculus region of gut and abnormal arrangement of middle cells of midgut in *dve*^{1} homozygous mutants (Fuß and Hoch 1998) (Nakagoshi et al. 1998). Dve is a vertex-specific dorsal selector gene, with its expression domain restricted to dorsal head capsule (vertex) region (Kiritooshi et al. 2014). Loss-of-function clones of *dve* in the head region results in ectopic antenna formation (Kiritooshi et al. 2014) and dorsal eye enlargements (Puli and Singh submitted). Human ortholog of *dve* is SATB-1 (special A-T rich binding sequences-1). SATB-1 is a matrix associated DNA binding factor (Dickinson et al. 1992) and is known to play crucial role in cervical cancers, esophageal cancers, etc. Dve also plays an important role in wing imaginal disc development (Kölzer et al. 2003). *dve* is crucial for the development of proximal-distal (PD) axis of the wing (Kölzer et al. 2003). The process of early development and specification of head capsule is conserved from species to species in *Drosophila* but variations in different head structures still have been seen in many species of *Drosophila like* lateral extensions of head capsules (a condition known as hypercephaly) in diopsid stalk-eyed flies as compared to normal head formation as seen in *Drosophila* (Carr et al. 2005).

Wg, a homolog of mouse Wnt-1 gene (Rijsewijk et al. 1987), is a secretory protein and a morphogen. Wg is expressed along the antero-lateral margins of the third instar eye imaginal disc (Fig. 7h, h') (Baker 1988a). 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 (Chang et al. 2001; Cho et al. 2000; Maurel-Zaffran and Treisman 2000). During the 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, which acts as a negative regulator of eye during retinal differentiation, also functions as a dorsal eye fate gene. In the dorsal eye imaginal disc, a N ligand, Dl has been assigned to the dorsal gene category (Table 2). Dl is preferentially expressed in dorsal domain of eye imaginal discs during first and second instar stages (Cho and Choi 1998). Apart from genes with domain-specific expression, there are genes which although expressed in broader domains but exhibits DV domain-specific functions (Table 2).

Asymmetrically Expressed Genes Regulating Domain-Specific Growth

A group of DV patterning genes exhibits differential functions in the dorsal-ventral compartments even though they are not expressed in a DV-specific pattern. The members of this group are further classified into three categories: (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 *homothorax* (*hth)* (Fig. 7e, e′; Table 2). (2) Genes that are expressed uniformly in the early eye imaginal disc and function differently in the dorsal and ventral half of the eye, for example, *teashirt* (*tsh)* (Fig. 7f, f′; Table 2; (Singh et al. 2004, 2005b, 2012), (3) Class of genes expressed in a domain-specific manner and are involved in generating morphogen gradient across the developing eye imaginal disc. They are (a) Marginally expressed genes like *optomotor blind (omb)* (Tare et al. 2013b) (Fig. 7g, g′; Table 2); Wg (Fig. 7h, h′; Table 2) and (b) Equatorially expressed genes like *four jointed (fj)* (Fig. 7i, i′; Table 2) and *unpaired (upd)* (Fig. 7j, j′; Table 2).

(1) Homothorax (Hth) is a vertebrate homolog of murine proto-oncogene MEIS1 (myeloid ecotropic viral integration site 1) (Moskow et al. 1995). It encodes a homeodomain transcription factor of the three-amino-acid extension loop (TALE) subfamily (Rieckhof et al. 1997). The expression of *hth* is present in the entire early eye primordium (Bessa et al. 2002; Singh et al. 2002, 2012), which is similar to *L* expression in the early eye (Singh et al. 2012). However, unlike *L*, which is uniformly expressed in the entire eye imaginal disc during all stages of eye development (Singh and Choi 2003), *hth* expression evolves with the onset of differentiation in the eye. Hth expression gets restricted to the cells anterior to the MF (morphogenetic furrow) (Bessa et al. 2002; Pai et al. 1998; Pichaud and Casares 2000; Singh et al. 2002). Although *hth* is expressed anterior to the furrow, its expression is uniform both in the dorsal and ventral half of the eye imaginal disc (Fig. 5a, a′; 7e, e′; Table 2). *hth* is expressed uniformly in the PM of the eye imaginal disc (Fig. 5a′). Surprisingly, the loss-of-function phenotypes of *hth* are restricted only to the ventral

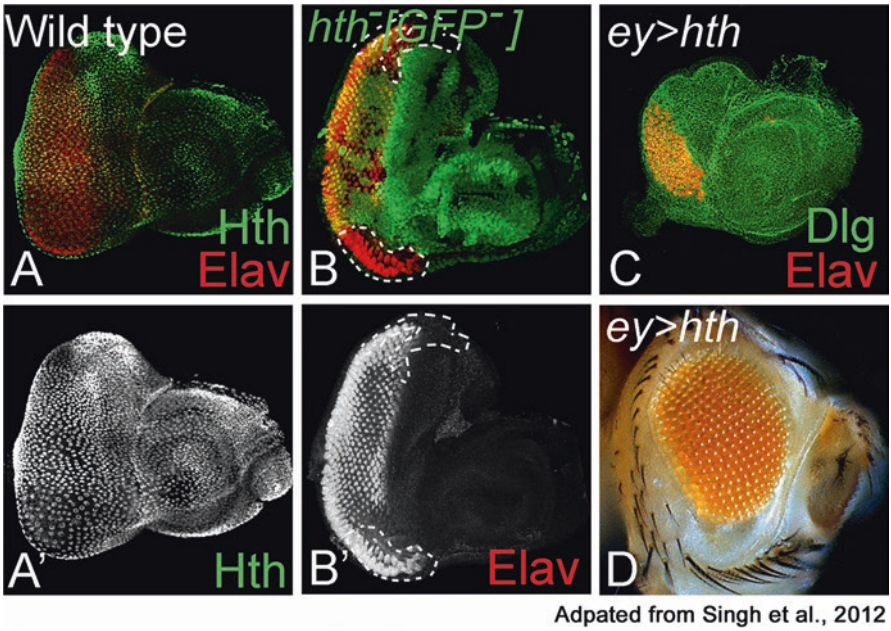

Fig. 5 A domain-specific function of *homothorax (hth)* in the ventral eye margin. (**a**, **a**′) Hth (green) is expressed anterior to the furrow both in the dorsal and ventral domain of the eye imaginal disc (Bessa et al. 2002; Pai et al. 1998; Pichaud and Casares 2000; Rieckhof et al. 1997; Singh et al. 2002). Elav (red), a pan-neural marker, marks the photoreceptor 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 (green, clonal boundary marked by white dotted line) in the ventral eye result in eye enlargements, whereas in the dorsal eye these clones do not have any effect. (**c**, **d**) Misexpression of *hth* in the eye using *ey*-Gal4 driver (*ey* > *hth*) results in a reduced-eye field as seen in the (**c**) eye imaginal disc and the (**d**) adult eye (Pai et al. 1998)

eye margins (Pai et al. 1998). Loss-of-function clones of *hth* causes eye enlargement only in the ventral eye margin (Fig. 5b, b′), whereas the loss-of-function clones of *hth* in the dorsal compartment do not show any phenotype in the eye imaginal disc (Pai et al. 1998; Pichaud and Casares 2000; Singh et al. 2011, 2012). Furthermore, *hth* mutant cells do not survive in the anterior eye (Bessa et al. 2002, 2008; Pichaud and Casares 2000). Therefore, despite the uniform expression of *hth* in developing eye imaginal disc the loss-of-function phenotype exhibits DV constraint. Misexpression of *hth* in the eye imaginal disc suppresses the eye fate (Pai et al. 1998). Furthermore, eye suppression function of Hth is independent of any domain constraint (Singh et al. 2011, 2012). During development, *hth* is involved in multiple functions 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) (Kurant et al. 1998; Noro et al. 2006; Pai et al. 1998; Rieckhof et al. 1997). Alternative splicing is known

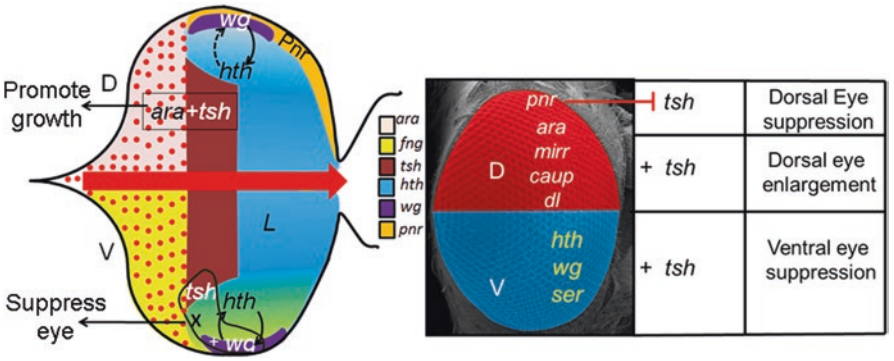

Fig. 6 Dorso-ventral asymmetric function of homeotic gene *tsh* depends on its partners (genes) in D and V compartment of the developing eye imaginal disc. Gain-of-function of *tsh* suppresses the eye fate in the ventral eye (Singh et al. 2002). Tsh collaborates with Wg and Ser and is required for suppression of the ventral eye. However, this eye suppression function of *tsh* in ventral eye is independent of genes *L* and *fng*. Dorsal eye enlargement function of *tsh* depends on collaboration of members of Iro-C family and the N ligand (Singh et al. 2004). In the dorsal eye, *pnr* is required to suppress *tsh* in order to suppress the dorsal eye fate (Oros et al. 2010)

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 (Jaw et al. 2000; Ryoo et al. 1999; Stevens and Mann 2007). Since Exd is expressed uniformly in the eye, the ventral eye specific function of *hth* has been proposed through its interaction with Wg and Tsh (Fig. 6). Together they are involved in suppression of eye fate on the ventral margin. Furthermore, *hth* plays an important role in delineating the boundary between the eye and the head cuticle on the ventral eye margin (Singh et al. 2011, 2012). We have found that *hth* antagonizes another ventral gene *L* function in the ventral eye margins to define the developing eye field boundary (Singh et al. 2011, 2012). However, this antagonizing activity is independent of Exd (Singh et al. 2011).

(2) The homeotic gene *tsh* belongs to the second category. It is expressed uniformly in the early eye imaginal disc but its function exhibits DV domain constraint. Tsh encodes a C_2H_2 zinc finger transcription factor with three widely spaced Zinc finger domains (Fasano et al. 1991). Tsh plays an important role during *Drosophila* eye development (Bessa et al. 2002; Datta et al. 2009; Kumar 2009, 2011; Pan and Rubin 1998; Singh et al. 2002, 2012). *tsh* is expressed anterior to the furrow both in dorsal and ventral eye (Fig. 7f, f′) and exhibits a DV constraint in its function (Fig. 6; Table 2) (Singh et al. 2002, 2004). In the dorsal eye, *tsh* promotes eye development, whereas in the ventral eye it acts as repressor of eye fate (Singh et al. 2002, 2004, 2005b, 2012). Interestingly, the DV constraint in *tsh* function in the eye depends on the partners with which it collaborates in the dorsal or the ventral eye disc (Singh et al. 2004). Tsh cooperates with Iro-C members and N ligand Dl in the dorsal eye for its growth promotion function (Singh et al. 2004). The ventral eye

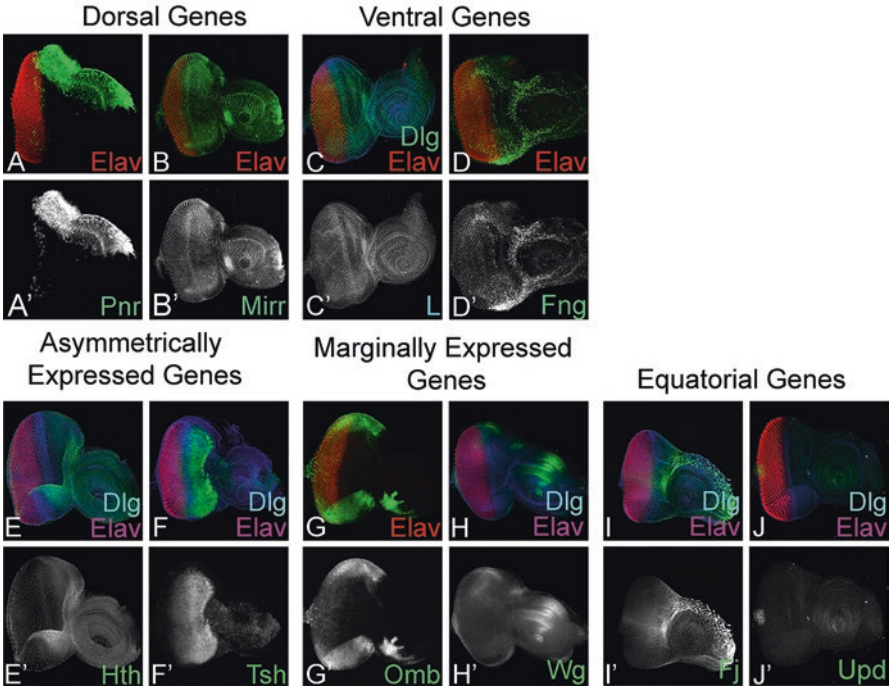

Fig. 7 Expression profiles of different genes required for axis determination during eye organogenesis. (**a**, **a**′) Pnr and (**b**, **b**′) Mirr (Green) are expressed in the dorsal domain of the eye. (**c**, **c**′) L is expressed ubiquitously in the entire eye imaginal disc (blue), whereas Fng (**d**, **d**′; green) is expressed only in the ventral domain of the eye. In the images **a**–**d**, Elav (red), the pan-neuronal marker is used for marking the photoreceptor neurons. Among asymmetrically expressed genes, Hth and Tsh (Green, **e** and **f**); (**e**, **e**′) Hth is expressed in an asymmetric fashion anterior to the morphogenetic furrow in the head and antenna but not in the eye region. (**f**, **f**′) DV asymmetric gene Tsh is also expressed anterior to the furrow both in dorsal and ventral domains of the eye and is regulated differentially in these regions. The eye imaginal discs in **e** and **f** have been stained for membrane marker Dlg (Blue) and pan-neuronal marker Elav (Magenta). (**g**, **g**′). Among marginally expressed, Omb and Wg (green; **g**, **g**′**, h,** **h**′) is expressed exclusively on dorsal and the ventral margins. (**h**, **h**′) Wg, a secreted morphogen is expressed along dorso-lateral margins and in the antenna (green). Equatorial genes are expressed on equator or the borderline of dorsal and ventral compartments. (**i**, **i**′) Fj (green) forms a gradient which is more concentrated on the equator and closer to the antennal region. (**j**, **j**′) Upd (green), the JAK STAT ligand is expressed on posterior boundary as a dot on the equator of the eye

specific function of *tsh* 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. 2002). 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 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 co-express in the proliferating cells anterior to furrow to block precocious retinal differentiation and promote cell proliferation (Bessa et al. 2002; Dominguez and Casares 2005; Singh et al. 2002). The role of *tsh* in the dorsal eye was further validated by studies on interaction of *tsh* with the dorsal fate selector *pnr* (Fig. 6) (Oros et al. 2010). It was shown that *pnr* suppresses the eye fate on the dorsal eye margin by downregulating *tsh* expression in the dorsal eye (Oros et al. 2010). Tsh is known to act upstream of retinal differentiation genes eyes *absent (eya), sine oculis (so), and dacshund (dac)* (Pan and Rubin 1998). Thus, *pnr* which is expressed in the dorsal PM (Fig. 7a, a′) suppresses *tsh* in the dorsal eye. It results in the suppression of eye fate on the dorsal margin of the eye field (Oros et al. 2010; Singh et al. 2012).

(3) During the patterning and growth of a field, the positional information or polarizing signals are provided in concentration-dependent manner. These signals are determined by concentration of signaling molecules or morphogens. Genetic studies of the polarity genes in *Drosophila* suggested that planar polarity in the dorsal and ventral eye fields is dependent on gradients of the polarizing signals (Wehrli and Tomlinson 1998; Zeidler et al. 1999b). It has been shown that non-canonical Wg/Wnt pathway is important for determining planar polarity (Boutros et al. 1998, 2000; Mlodzik 1999; Reifegerste and Moses 1999; Singh et al. 2005b). In the developing *Drosophila* eye, Wg is responsible for the pole to equator gradient (Legent and Treisman 2008; Zecca et al. 1996). In the third instar eye imaginal disc, Wg is strongly expressed on the antero-lateral margins (Fig. 7h, h′; Table 2). It results in a diffusible pole to equator gradient that originates from the dorsal and ventral margins of the eye disc epithelium (Fig. 7h, h′). It suggests that Wg can function as a primary polarizing signal. Since Wg is involved in other events during eye development and loss of *wg* causes defects in the eye (Wehrli and Tomlinson 1998), it may not suffice to state that Wg is strictly required only for planar polarity. It is possible that DV patterning in the eye also requires the Wg gradient to interpret patterning cues in the developing eye imaginal disc. The local cues within the cell are crucial for the Wg gradient but the secondary cell interactions shape the morphogen gradient by interpreting the information and setup differential expression.

Another candidate which may be participating in pole to equator gradient can be the T-box transcription factor Omb, also known as bifid (bi). Omb is a target of Wg signaling in the wing (Zecca et al. 1996). Its expression is regulated in the wing by Dpp and Wg. In the eye imaginal disc epithelium, *omb* is expressed in an equator to pole gradient where it has highest level on the dorsal and ventral margins and its levels decrease towards the equator (Fig. 7g, g′, Table 2) (Tare et al. 2013b). It is also expressed in some glial cells (Poeck et al. 1993). Gain-of-function of *omb* results in the reduction of eye size and loss-of-function of *omb* exhibits enhanced proliferation in the ventral eye disc (Porsch et al. 2005). Omb functions to delimit the extent of the DV eye (Poeck et al. 1993). The insertion of a P-element carrying a *white*$^{+}$ gene in the *omb* locus results in pigmentation on the dorsal and ventral eye

margins in the adult eye. An *omb*-Gal4 line that was later isolated by Calleja and colleagues and Tang and Sun has been used vastly as an important tool to drive expression of genes on the dorsal and ventral border margins of the eye imaginal disc (Calleja et al. 1996; Tang and Sun 2002; Tare et al. 2013b).

Another gene *extramacrochaetae (emc)* which encodes a helix-loop-helix (HLH) protein can bind to the basic HLH proteins, and form heterodimers (Alifragis et al. 1997; Ellis et al. 1990; Garrell and Modolell 1990; Van Doren et al. 1991). Emc protein lacks DNA binding domain and therefore cannot interact with DNA both in its original form or as heterodimers (Van Doren et al. 1991). Loss-of-function (LOF) of *emc* results in developmental defects (Bhattacharya and Baker 2009; Brown et al. 1995). It has been reported that *emc* play a crucial role in DV patterning. Wg, a negative regulator of eye development, can prevent initiation or formation of ectopic morphogenetic furrow (Ma and Moses 1995; Treisman and Rubin 1995). Interestingly, LOF of *emc* also results in formation of ectopic MFs while GOF of *emc* blocks ectopic MF formation at both dorsal and ventral domains of eye (Spratford and Kumar 2013). The possible explanation for this different behavior of *emc* in the dorsal and the ventral domains could be that Emc and Wg act independently or parallel and prevent ectopic MF formation in the dorsal margin, whereas in the ventral margin, Emc works together with Wg and regulates its transcription (Spratford and Kumar 2013). *emc* is expressed along the midline (ahead of MF) in the third instar larval eye-antennal imaginal disc (Bhattacharya and Baker 2009; Brown et al. 1995; Spratford and Kumar 2013). Emc acts downstream of Notch (N) signaling and its expression can be seen both at early or later stages of DV patterning in *Drosophila* eye (Spratford and Kumar 2015).

In addition to pole to equator gradient of Wg, Fj, a Golgi-associated protein and a member of the planar cell polarity pathway (PCP pathway), has been proposed to be involved in equator to pole signaling (Zeidler et al. 1999a). It is expressed in a broad equatorial domain (Fig. 7i, i′) (Brodsky and Steller 1996; Heberlein et al. 1998). The Fj expression gradient provides directional cues in ommatidial polarity (Zeidler et al. 1999a). Its gradient of expression is highest at the equator and decreases towards the margins. This graded expression of Fj is opposite to that of the pole to equator gradient of Wg, Omb, and Dachsous (Ds), which are highest at the dorsal and ventral margins (poles) of the eye imaginal epithelium and decreases towards the equator. Fj acts upstream to Ds, therefore modulating and restricting its gradient expression.

Upd, a ligand of the JAK/STAT pathway (Harrison et al. 1998) is also required in the developing eye (Zeidler et al. 1999b). In the *Drosophila* eye, Upd is expressed at the posterior margin of the eye disc and is required to repress Wg and to promote differentiation in the eye (Table 2) (Singh et al. 2012; Tsai and Sun 2004; Tsai et al. 2007). Therefore, Wg downregulates Fj and N and Upd acts as positive regulators of Fj (Reynolds-Kenneally and Mlodzik 2005; Zeidler et al. 1999a). Fj and Upd, together or in parallel, are candidates for the secondary signal. These studies suggest that DV patterning genes not only contribute towards the growth of the eye field but also in delineation of boundary between the eye and head field.

Boundary Formation During Organogenesis

One of the important questions is how DV patterning genes regulate size and growth of the eye as an organ. The dorsal selector genes like *pnr, Iro-C* members, which are expressed in the dorsal eye margin (Fig. 7a, b), assign a dorsal fate in a group of early eye primordial cells that are basically ventral in fate. These dorsal fate selectors generate a group of dorsal cells with unique properties. The boundary between the dorsal and ventral cells (equator) is maintained by the antagonistic interactions between the dorsal and ventral eye genes (Singh et al. 2005a). It has been shown that *L/Ser* 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 (Fig. 8). 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. 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* (Fig. 8). It has been suggested that *pnr* is required for two different functions during eye development: (a) DV axis determination during early eye development (Maurel-Zaffran and Treisman 2000; Singh and Choi 2003) and (b) suppression of retinal determination to define the dorsal eye field margin (Oros et al. 2010; Singh and Choi 2003; Singh et al. 2005b, 2012) During early second instar of larval 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; Oros et al. 2010; Singh and Choi 2003; Singh et al. 2005b). However, during the late second instar stage of eye development *pnr* suppresses the photoreceptor differentiation at the dorsal eye margin (Oros et al. 2010; Singh et al. 2012). The expression of *pnr* is restricted to the peripodial membrane of the dorsal eye margin, which gives rise to the adult head cuticle. Loss-of-function clones of *pnr* exhibit dorsal eye enlargement. It suggests that absence of *pnr* function promotes ectopic eye formation in the dorsal eye margin. Therefore, *pnr* defines the boundary between the head cuticle and the dorsal margin of the developing eye field (Oros et al. 2010; Singh et al. 2012). There is a different mechanism for delineating the boundary between the eye and head cuticle on the ventral eye margin as *pnr* is not expressed in the ventral eye (Singh et al. 2011). The boundary of eye field on the ventral eye margin is defined by the antagonistic interaction of *L* with *hth* (Singh et al. 2011, 2012). Antagonistic interaction between L and Hth is not the exclusive mechanism to define the ventral eye margin. In the ventral eye, transcriptional co-factor Chip interacts with the LIM homeodomain proteins to define the boundary of the eye field (Roignant et al. 2010). Interestingly, Chip-mediated regulation of the ventral eye boundary is independent of *hth* (Roignant

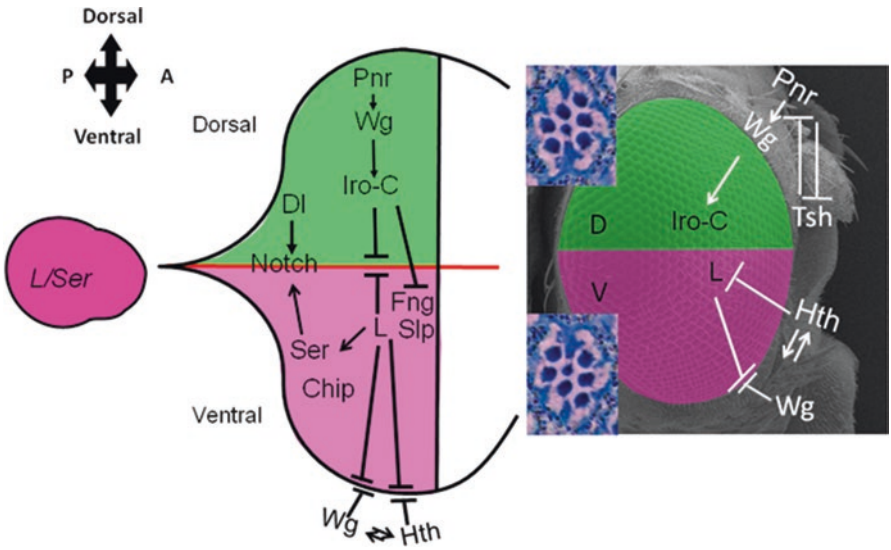

Fig. 8 Genetic basis of DV asymmetry in the developing *Drosophila* eye. (**a**) During early eye development (first instar larva), the entire eye primordium belongs to a homogenous ventral state assigned by functions of *L/Ser.* (**b**) In later stages (second instar and early third instar stages), dorsal lineage is specified upon onset of *pnr* expression. *pnr* acts upstream of *wg* and this interaction is required for triggering expression of downstream genes like members of Iro-C complex (Maurel-Zaffran and Treisman 2000). Dl, a ligand of N pathway, is also required for the development of the dorsal eye. The default state of ventral eye is maintained by expression of *L/Ser*. *L* has been shown to antagonize functions of genes in the dorsal eye to define the eye boundary between dorsal and ventral compartment of the eye. Furthermore, functions of *L/Ser* are also required for antagonizing Hth and Wg and define the ventral eye margin to prevent cell death, respectively (Singh et al. 2011). However, there is a positive feedback loop between Hth and Wg in the ventral eye margin. The other genes important for ventral eye development are *chip*, *fng,* and *slp* (Cho and Choi 1998; Dominguez and de Celis 1998; Legent and Treisman 2008; Roignant et al. 2010; Sato and Tomlinson 2007). The interactions of dorsal and ventral genes are responsible for structural and functional organization of the adult compound eye of the *Drosophila*. (**c**) The ommatidia in an adult compound eye are organized into mirror image symmetry which are polarized into opposite directions of dorsal and ventral half

et al. 2010). Thus, the genetic cascade regulating the boundary of eye field on the dorsal and the ventral margin of the eye is different.

Proximo-distal Axis in the Eye

Most flies including *Drosophila melanogaster* have their compound eyes and antenna located in a socket on the adult head (Fig. 9a, b). Therefore, there is no distinct proximo-distal (PD) axis defined in the *Drosophila* eye. However, other appendages like wing, antenna, and leg have distinct proximo-distal axis. From evolution

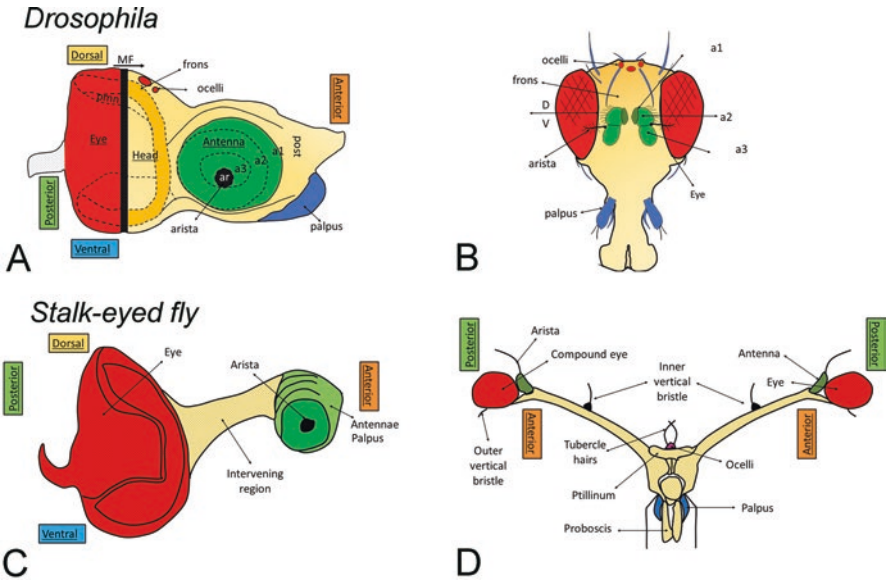

Fig. 9 Cartoons comparing eye development in *Drosophila melanogaster* and the *stalk-eyed fly*. (**a**) *Drosophila* third instar eye-antennal imaginal disc that develops into the (**b**) adult head of the fly. Note that the adult compound eye is present in a socket on the adult head and does not have any distinct proximo-distal (PD) axis. (**c**) Stalk-eyed fly eye-antennal imaginal disc which develops into (**d**) adult head. The adult compound eyes and antenna of stalk-eyed fly are located on a lateral extension from the adult head. These structures exhibit a distinct PD axis

standpoint, eye development is a relatively new trait. Interestingly, morphological diversity is an outcome of modification of body plans due to changes in development programs during evolution. The stalk-eyed flies from Diopsidae family exhibit a deviation where the antenna, eye, and optic lobe located at the end of the stalk (Fig. 9c, d) (Buschbeck and Hoy 2005; Buschbeck et al. 2001). The length of the stalk or eye span, a sexually dimorphic trait, varies among different dipteran species (Baker et al. 2001; Buschbeck et al. 2001). The stalk length plays an important role in the selection of a male mating partner, where males with longer stalks have an advantage over other males (Cotton et al. 2014; Wilkinson and Reillo 1994). This "stalk-eyed" morphology is a dramatic deviation from other dipterans, including *Drosophila* (Buschbeck and Hoy 1998). In these stalk-eyed flies, there is a distinct PD axis delineation involved in the eye development regimen (Singh et al. 2019). The presence of an intervening region of proximal fate in larval eye-antennal imaginal disc provides a basis for this distinct hypercephalic phenotype observed in the adult fly.

Similarities with Vertebrate Eye

There are remarkable similarities in general developmental design based on functional and structural homologies between the *Drosophila* eye genes and the vertebrate *e*ye *f*ield *t*ranscription *f*actor*s* (EFTFs) (Wawersik and Maas 2000). Furthermore, the basic sensory epithelium design of the vertebrate and most invertebrate eyes including the *Drosophila* eye is similar (Charlton-Perkins and Cook 2010; Sanes and Zipursky 2010; Singh et al. 2012). The morphogenetic furrow (MF) in the fly eye is analogous to the wave of neurogenesis in the vertebrate retina (Hartenstein and Reh 2002; Neumann and Nuesslein-Volhard 2000). However, the MF in the *Drosophila* eye initiates from the posterior margin and moves towards the anterior part of the developing eye imaginal disc, whereas differentiation in vertebrate retina initiates from center and moves radially out (Hartenstein and Reh 2002). Several genes that are expressed in a DV domain-specific manner in the retina have been identified in the vertebrate visual system. Transcription factors such as Pax-6, Pax-2 (*ey* in *Drosophila)* along with Sonic hedgehog (Shh) signaling (Hh in *Drosophila*) have been known to regulate DV patterning during eye development in vertebrates (Saha et al. 1992). LOF of Pax-6 and Pax-2 results in no eye or small eye phenotypes (Fujiwara et al. 1994; Hill et al. 1991) or defective eye phenotypes in mouse and rats. (Torres et al. 1996) In the dorsal half of the eye, BMP4, a TGF-β closely related to Dpp, has been implicated in development of progenitor cells. It has also been shown to function in establishment of the DV axis of the Xenopus retina (Papalopulu and Kintner 1996). In the vertebrate eye, the dorsal selectors BMP-4 and TbX5 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). The DV patterning plays an important role in the 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). Jagged-1(Jag1), a vertebrate homolog of the *Drosophila* ventral eye gene *Ser*, exhibits a DV asymmetric expression pattern in the retina. In addition, loss-of-function of Jag1 results in Alagille's syndrome, which also affects the eye (Kim and Fulton 2007; Oda et al. 1997; Xue et al. 1999). It has also been suggested 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 the 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.

Drosophila melanogaster is a highly versatile, genetically tractable model system to study biological phenomenon (Bellen et al. 2010; Bier 2005). Our understanding of molecular genetic mechanisms of fundamental processes of patterning (axial patterning), growth, cell death, cell survival in developing *Drosophila* eye has

allowed the use of *Drosophila* eye as a model to study human disease (Cutler et al. 2015; Fernandez-Funez et al. 2013; Irwin et al., 2020; Gogia et al., 2020; Yeates et al., 2019). Nearly 75% of human disease causing genes have their functional homologs in flies. The extent of structural and genetic similarity of *Drosophila* and human eyes allows exploitation of this model to study human disease. Furthermore, the *Drosophila* eye phenotypes are easy to score and thus allow genome wide, chemical screens or screen other natural products to understand and find cure for the disease (Cutler et al. 2015; Deshpande et al. 2019; Sarkar et al. 2018b).

Summary

In this book chapter, we have focused on the key developmental events and genes that are involved in DV patterning of the *Drosophila* eye. It has been established that formation of the DV compartment formation is a key event in initiating patterning and growth of the early eye imaginal disc. This may also hold true in primordia of other adult appendages. It is clear that DV patterning is required to initiate the DV asymmetry within a homogenous default ventral fate of early eye primordial cells. Even though our understanding of the DV patterning in the eye has dramatically increased in recent years, our understanding of the axial patterning of the *Drosophila* eye is far from complete, and we are still not aware of all the members of genetic circuitry and the molecular interactions between them which are important for the regulation of DV patterning. There is a room for identification of many more novel genes that are involved in DV patterning. The future studies using novel genetic and bioinformatics approaches should help in defining the full complement of genes involved in this intricate process. These studies will help in addressing the age-old question of how a small number of cells in the disc primordium grow to form a precise pattern of mirror symmetry in the compound eye. In addition, the possibility of crosstalk of the DV patterning pathway with other signaling pathways to regulate growth during early phase of eye development cannot be refuted. 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 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.

Acknowledgements The authors thank Dr. Meghana Tare and Aditi Singh for their help and comments on the manuscript. We apologize to all authors whose work could not be cited due to space limitations. AS is supported by NIH1R15GM124654-01 from NIH, Schuellein Chair Endowment Fund and STEM Catalyst Grant from the University of Dayton to A.S.

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Catching the Next Wave: Patterning of the Drosophila Eye by the Morphogenetic Furrow

Justin P. Kumar

The Adult Eye: A Product of Pattern Formation

The compound eye of *Drosophila* is a simple nervous system of such extraordinary precision that it has been described as a "neurocrystalline lattice" (Fig. 1; Ready et al. 1976). The adult retina consists of approximately 800 unit eyes or ommatidia that are organized into nearly three dozen vertical columns. Each unit eye is constructed as a hexagon, thus adjoining columns of ommatidia appear to be interlocked with each other. The number of unit eyes per column is variable with columns in the center of the eye containing the largest number of ommatidia while those that lie at increasing distances from the center have fewer and fewer unit eyes. This arrangement gives the compound eye an overall egg or oval shape. During larval development, the first column, which is set down at the posterior margin of the retina, serves as a template upon which the next ommatidial column is added. Subsequent columns are similarly added, with each preceding column serving as a template for the next, until the approximately three dozen columns of unit eyes are set within the eye primordium. It is the responsibility of the morphogenetic furrow to inlay each column of ommatidia onto the epithelium (Fig. 2b; Ready et al. 1976; Lebovitz and Ready 1986; Wolff and Ready 1991).

Each unit eye consists of eight photoreceptor neurons, four lens secreting cone cells, and a cadre of optically insulating pigment cells (Fig. 3c; Dietrich 1909; Waddington and Perry 1960; Ready et al. 1976; Tomlinson and Ready 1987; Cagan

A Drosophila third instar eye-antennal disc (green) stained with antibodies that detect the expression of the Lim1 (yellow) and Cut (red) transcription factors. The disc was photographed by Bonnie M. Weasner and Justin P. Kumar.

J. P. Kumar (✉)
Department of Biology, Indiana University, Bloomington, IN, USA
e-mail: jkumar@indiana.edu

A. Singh, M. Kango-Singh (eds.), *Molecular Genetics of Axial Patterning, Growth and Disease in Drosophila Eye*,
https://doi.org/10.1007/978-3-030-42246-2_3

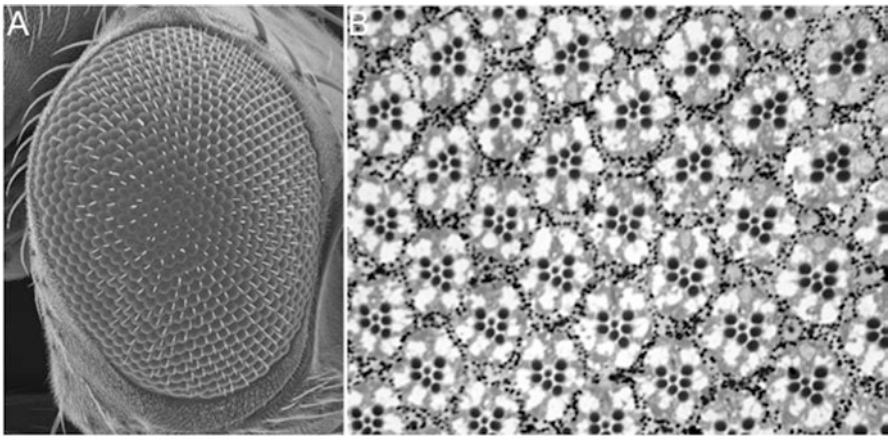

Fig. 1 Structure of the adult compound eye of Drosophila: External view and retinal section. (**a**) Scanning electron micrograph of the adult compound eye. Note the roughly three dozen columns of unit eyes or ommatidia. (**b**) Light microscope section of the adult retina. The photoreceptors are organized into an asymmetrical trapezoid. A line of mirror symmetry (the equator) divides the eye into dorsal and ventral compartments. The ommatidia on either side of the equator exist in two chiral forms. Anterior is to the right in all images.

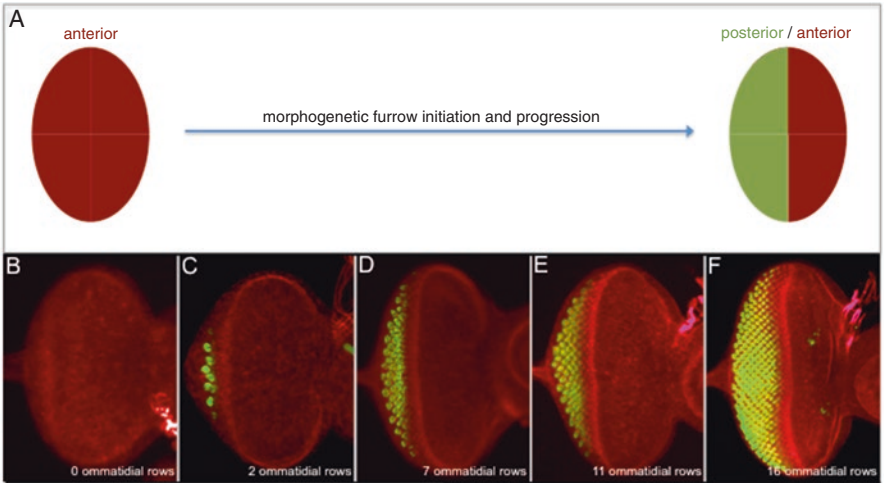

Fig. 2 Progression of the furrow transforms a field of undifferentiated cells into an organized array of unit eyes. (**a**) Schematic depiction of the anterior compartment boundaries in the developing eye. During early development, the entire eye is made up of anterior fated tissue (red). After the furrow initiates and progresses across the eye field, the eye is slowly converted into all posterior tissue (green). The schematic shows an eye in which the furrow has migrated across half of the epithelium. (**b**–**f**). Confocal images of third instar eye discs in which the furrow has progressed to various points. The number of ommatidial rows is shown in each panel. Red = F-actin and green = Elav. Anterior is to the right in all panels

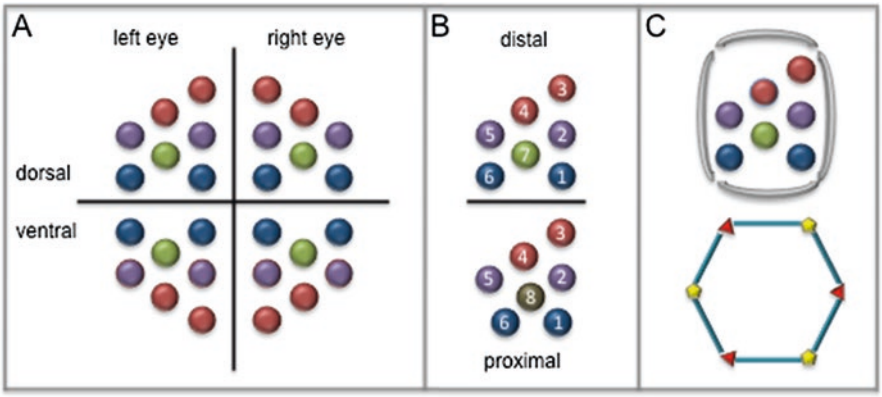

Fig. 3 Schematic of cell types and orientation within the adult retina. (**a**) Schematic depicting the different chiral forms that are found in the four quadrants of the two adult compound eyes. (**b**) Schematic depicting the photoreceptors that are found in the distal and proximal layers of the retina. The identity of each photoreceptor is listed within the figure. (**c**) Schematic depicting the different cell types that are present within each ommatidium. In the top portion of the panel, the round circles and grey brackets represent the photoreceptor neurons and cone cells, respectively. In the lower portion of the panel, the blue bars are the secondary pigment cells, the red triangles are the tertiary pigment cells, and the yellow hexagons are the mechanosensory bristles. Anterior is to the right in all panels

and Ready 1989a). The photoreceptors lie at the core of the ommatidium and are arranged as an asymmetric trapezoid. Ommatidia exist in two chiral forms with the equator serving as the transition point between the two forms. Ommatidia in the dorsal half of the retina point "north" while those in the ventral half point "south" (Fig. 3a). The outer photoreceptors R1–6 occupy the entire length of the ommatidium while the inner neurons R7/8 reside with the distal and proximal sections, respectively (Fig. 3b). The cone cells lie atop the photoreceptor cluster and secrete the overlying lens while the pigment cells surround and optically insulate the photoreceptors. Their physical arrangement gives the ommatidium its hexagonal shape. The furrow not only organizes the eye into columns of unit eyes, it also contributes to the earliest step in ommatidial assembly—the specification and recruitment of the R8 photoreceptor (Figs. 8, 11; see discussion below). The specification of the R8 then begins the recruitment of the remaining photoreceptors, cone cells, and pigment cells (Fig. 8; reviewed in Kumar 2012).

Early Eye Development: A Prologue to Furrow Initiation

The origins of the adult retina can be traced back to the optic primordium of the embryonic blastoderm when approximately twenty cells are set aside to develop independently from the rest of the embryo (reviewed in Cohen 1993; Held 2002). While these cells are morphologically indistinct and fail to express any tissue-

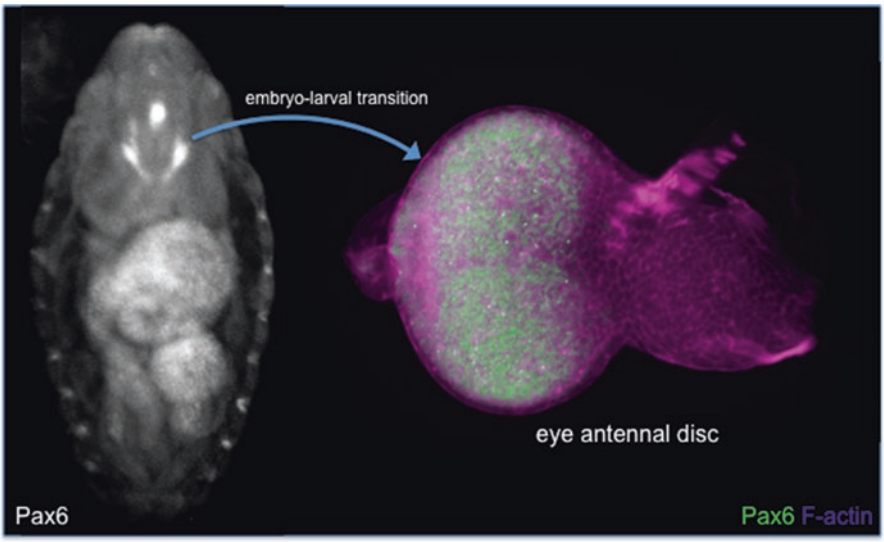

Fig. 4 Birth of the eye-antennal disc. (Right portion) Confocal image of a stage 16 embryo in which the eye-antennal disc is visualized by the presence of the Ey/Pax6 protein. Note that Ey is distributed throughout the entire eye disc. Anterior is to the top in this panel. (Left portion) Confocal image of a mid/late second instar eye-antennal disc. Note that by this point Ey protein is segregated to just the eye portion of the epithelium. Visualized molecules are listed within the images. Anterior is to the right in this image

specific genes at these early times, their existence and ancestry have been confirmed through fate mapping experiments (Struhl 1981). Midway thru embryogenesis the eye anlage is fused to the antennal primordium to form a single monolayer epithelium called the eye-antennal disc (reviewed in Jurgens and Hartenstein 1993). Shortly after this fusion, the eye-antennal disc can be identified by the combined expression of several markers (Fig. 4) including *escargot* (*esg*), which marks all imaginal discs, and several Pax6 genes such as *eyeless* (*ey*: Quiring et al. 1994), *twin of eyeless* (*toy*: Czerny et al. 1999); *eyegone* (*eyg*: Jones et al. 1998; Jun et al. 1998) and *twin of eyegone* (*toe*: Yao et al. 2008). By the first larval instar stage the eye portion of the disc is attached to the brain via the Bolwig's nerve, while the antennal segments are directly attached to the dorsal pouch (reviewed in Jurgens and Hartenstein 1993).

During the first and second larval instars, the eye field is primarily concerned with growth (reviewed in Kumar 2011). At the end of the second instar, the eye disc contains approximately 2000 cells, a 100-fold increase in size from its embryonic origins (Becker 1957). Since pattern formation via the morphogenetic furrow has thus far not initiated, all cells within the eye disc are still undifferentiated and not patterned at this point in development. Yet, some basic features of tissue organization can be discerned. For instance, the field itself has already taken on its characteristic oval shape and can be easily distinguished from all other imaginal discs. Additionally, by this stage the eye primordium, which was born with only ventral identity, is sub-

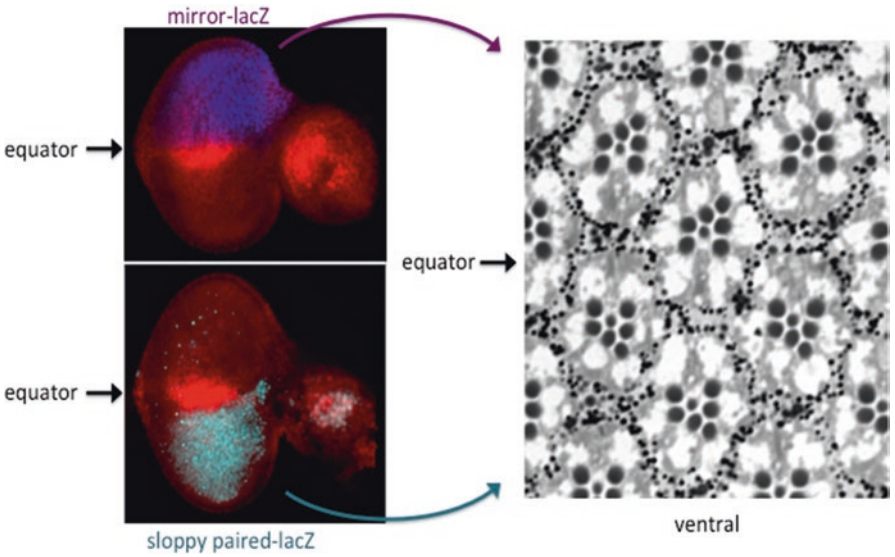

Fig. 5 The dorsal and ventral compartments are set prior to furrow initiation. Confocal images of mid/late second instar larval eye-antennal discs depicting expression of the mirror-lacZ (purple) and sloppy paired-lacZ (aqua blue) transcriptional reporters. The midline is marked by the expression of an emc-GFP (red) transcriptional reporter. The division of the eye into dorsal and ventral compartments early in development results in the adult eye containing different chiral forms of ommatidia. In the right portion of the figure, a light microscope section of the adult retina is shown. In this image, the different chiral forms are seen in the dorsal and ventral halves of the eye. Anterior is to the right in all images

divided into dorsal and ventral compartments (Fig. 5; Cho and Choi 1998; Dominguez and de Celis 1998; Papayannopoulos et al. 1998; Singh and Choi 2003). And finally, while cells within the disc have not taken on specific cellular identities the tissue itself is committed to adopting an eye fate as members of the retinal determination network which are first expressed broadly throughout the entire eye-antennal disc are segregated to just the eye field (Fig. 4; Kumar and Moses 2001a, b; Kenyon et al. 2003; Kumar 2010).

Cellular Architecture of the Furrow

Apical Constrictions and Tissue Ingression

At the transition to the third instar larval stage, overt patterning of the retina begins when the furrow initiates at the posterior margin of the eye primordium and proceeds towards the anterior edge where the eye and antennal fields meet (Fig. 2). As the name implies, the furrow is an actual physical groove in the epithelium. When

the developing retina is viewed in cross section, cells within the furrow have a bottle shape appearance and undergo a slight ingression (Fig. 6; Ready et al. 1976). Cell shape changes and invaginations of this kind are seen in a variety of tissues and it is thought that both are caused by the constriction of apical cell surfaces (Kimberly and Hardin 1998). Similar cellular events appear to be in play within the eye disc as cells that are approaching and entering the furrow undergo dramatic constriction of their apical profiles (Fig. 6b; Ready et al. 1976). Mechanistically, Hedgehog (Hh) signaling from developing photoreceptors induces apical surface constriction by triggering a reorganization of apical positioned microtubules and actin filaments (Heberlein et al. 1993; Benlali et al. 2000; Corrigall et al. 2007). This event is essential for the ordered development of the eye as mutations that interfere with apical cell constriction cause precocious neuronal development (Benlali et al. 2000).

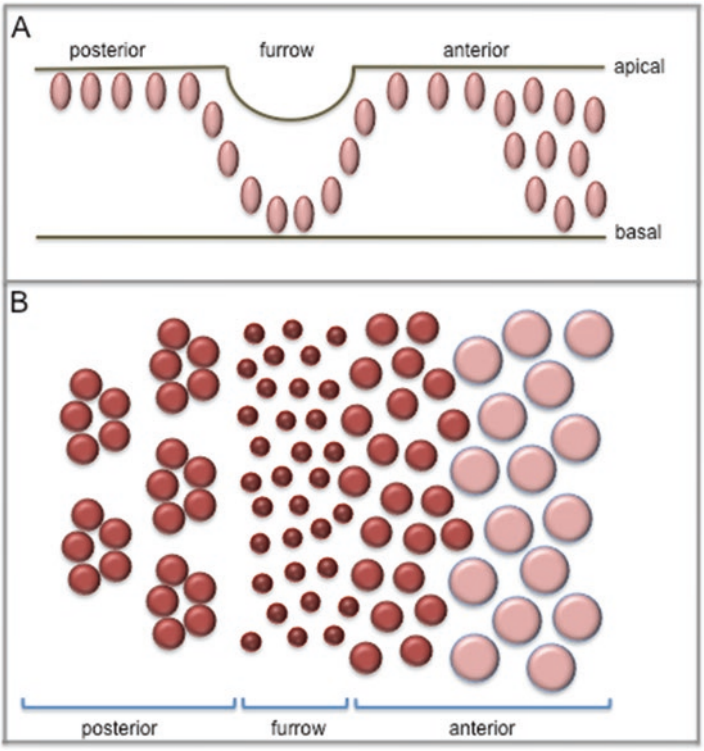

Fig. 6 Nuclear migration and apical constriction in the furrow. (**a**) A schematic depiction of the eye disc shown in cross section. Ahead of the furrow, nuclei are randomly distributed. As cells approach the furrow, their nuclei migrate to the apical surface. As cells enter the furrow, the nuclei plunge basally but ascend again as they exit the furrow and begin differentiation. (**b**) Schematic of the eye disc showing the apical profiles of cells ahead within and behind the furrow. As cells enter the furrow, their apical profiles are constricted. Behind the furrow, cells are organized into periodically spaced clusters—their apical profiles expand. Anterior is to the right in all images

How do changes in cell shape affect cell fate decisions? The answer to this question comes from an analysis of the nature of the Hh signaling itself. The Hh ligand is a signaling molecule that can function at both short and long ranges (Lee et al. 1992; Basler and Struhl 1994; Heemskerk and DiNardo 1994; Tabata and Kornberg 1994). In the retina it is expressed in and secreted from developing photoreceptor cells and influences patterning and cell shape changes over only a short range: just in a small stripe of approximately 10 cell diameters within and just ahead of the furrow (Heberlein et al. 1993; Ma et al. 1993). Restricting its range for patterning is conveniently accomplished by the constriction of apical profiles in cells just ahead and within the furrow. It is thought that this constriction results in a concentration of apical membrane, the site of the Patched (Ptc) receptor. This allows for the efficient capture of Hh molecules and prevents its signal from traveling too far ahead of the furrow. Thus cells lying just ahead and within the furrow receive the Hh signal, constrict their apical profiles, increase the capture of the ligand, and thereby prevent the signal from traveling further. This model is supported by the observation that the loss of *act up* (*acu*), which encodes the fly homolog of cyclase-associated protein (CAP), results in the retention of large apical profiles and an accumulation of the active form of Cubitus Interruptus (Ci^{ACT}), the zinc finger transcription factor that transduces the Hh signal, in cells that lie in more anterior regions of the disc than found in normal retinas (Benlali et al. 2000).

Nuclear Migration

The cell shape changes that are seen within the furrow are also influenced by the position of the nucleus. In anterior regions of the disc, nuclei are randomly positioned within the apical basal plane of the epithelium. Then, much like cars on a rollercoaster, nuclei will first rise to the apical surface, rapidly plunge to the basement of the epithelium and then ascend again. These nuclear movements are choreographed with the approach, entrance and exit of cells from the furrow (Fig. 6a; Ready et al. 1976; Tomlinson 1985). As in other developmental contexts, nuclear migration in the eye is dependent upon microtubules and the activity of the cytoplasmic motor protein Dynein (Fan and Ready 1997; Swan et al. 1999; Patterson et al. 2004; Houalla et al. 2005). The current model is that the KASH domain containing protein Klarsicht interacts with both nuclear Lamin and the microtubule-organizing center (MTOC) to physically link nuclei to microtubules (Patterson et al. 2004). Dynein is then thought to aid in the movement of the nucleus during its migration. It is not clear if the other major microtubule motor protein, Kinesin, is also involved in nuclear migrations within the eye.

The basal migration of nuclei within the furrow contributes to the bottle-like appearance of cells within this zone while the subsequent rise of nuclei as cells exit the furrow is coordinated with the adoption of individual cellular fates. While pattern formation appears to be coordinated with changes in nuclear position, is the former dependent upon the latter? The answer to this question is mixed. Cells that

comprise the pre-cluster (R8, R2/5, R3/4) appear to differentiate normally despite disruptions in nuclear migration and positioning. The only visible defects within these neurons are malformations of the rhabdomere, the light capturing organelle of the insect photoreceptor (Fischer-Vize and Mosley 1994; Welte et al. 1998; Mosley-Bishop et al. 1999). However, when nuclear positioning is disrupted within the second mitotic wave, differentiation of R1/6 and R7 fails to occur correctly (Fan and Ready 1997). It is unclear why such a difference exists between the two classes of photoreceptor neurons nor is it completely settled that the loss of the final three photoreceptors is due to nuclear positioning and not another yet to be described defect in microtubule dynamics.

Cell Cycle Synchronization

Another feature is the cell cycle synchronization of cells within and just ahead of the furrow. In the most anterior regions of the eye disc, cells are proliferating asynchronously and express markers for all four phases (G1, S, G2, and M) of the cell cycle (Fig. 7; Ready et al. 1976; Thomas et al. 1994; Richardson et al. 1995). However, just ahead of the furrow the cell cycle profiles begin to synchronize. Approximately ten cell diameters anterior to the furrow cells cease to express *cyclin E* (*cycE*) and fail to incorporate the thymidine analog 5-bromo-2-deoxyuridine (BrdU), thereby suggesting that all cells within this zone have progressed through and past S phase (Thomas et al. 1994, 1997). This is closely followed by the termination of *cyclin A* (*cycA*) and *cyclin B* (*cycB*) transcription, which signals the transition through G2. And finally, levels of *string* (*stg*), the fly homolog of yeast cdc25, are elevated in cells that are just about to enter the furrow (Edgar and O'Farrell 1989; Thomas et al. 1994). This last step signals cells to undergo a final mitosis before entering the morphogenetic furrow (Penton et al. 1997; Horsfield et al. 1998). Within the furrow all cells are arrested in G1 and do not express appreciable levels of the above discussed cyclins (Fig. 7).

Synchronization at G1 within the furrow is an important step as key decisions regarding cell cycle re-entry and exit are made shortly after cells leave the furrow. A subset of cells will exit the cell cycle and form periodically spaced clusters that contain five photoreceptors (Fig. 8; R8, R2/5, and R3/4). Any cell that does not exit and differentiate will re-enter the cell cycle, undergo one final round of division, and then give rise to the final three photoreceptors (R1/6 and R7), the lens secreting cone cells, and the optically insulating pigment cells (Fig. 8). As this is happening across the entire dorsal-ventral axis of the eye disc, it is important for all cells to have been arrested in G1 within the furrow, so that decisions regarding cell cycle exit and differentiation as well as cell cycle re-entry can be synchronized along the length of the furrow.

Given that pattern formation and cell cycle synchronization are coordinated within the furrow, a prime candidate for regulating G1 arrest is *decapentaplegic* (*dpp*), which encodes a Transforming Growth Factor-β family member (Padgett

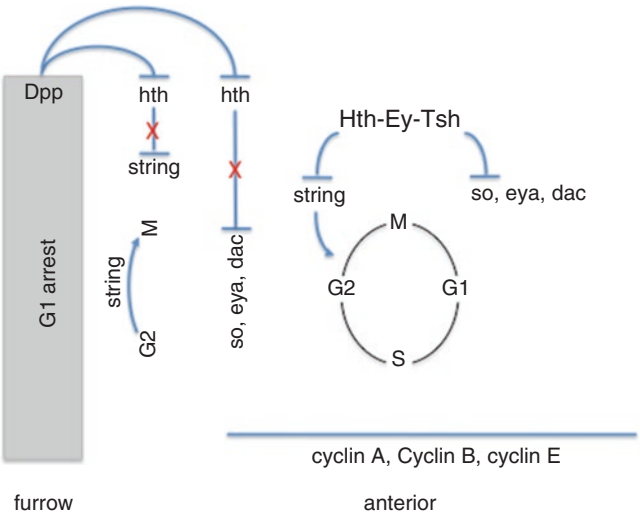

Fig. 7 Regulation of the cell cycle ahead and within the furrow. Schematic depicting the role that Dpp signaling plays in arresting cells in the G1 phase of the cell cycle. Far ahead of the furrow cells are dividing asynchronously. As cells approach the furrow, they simultaneously express *string* while ceasing to express *cyclin A*, *B,* and *E* and are thus arrested in G1 within the furrow. Dpp signaling counteracts the activity of Hth, which normally represses string transcription. Anterior is to the right

et al. 1987). The TGF-β superfamily has been implicated in the regulation of the cell cycle in a number of tissues and organisms (Massague and Polyak 1995). Prior to the initiation of pattern formation, *dpp* is expressed along the posterior margins of the eye disc and is essential for proper initiation of the furrow (Blackman et al. 1991; Heberlein et al. 1993; Chanut and Heberlein 1997a, b). In later stages, *dpp* expression is found exclusively within the furrow and is primarily tasked with repressing the expression of *wingless* (*wg*), a negative regulator of the furrow (Burke and Basler 1996; Dominguez and Hafen 1997; Greenwood and Struhl 1999; Curtiss and Mlodzik 2000). Mutations in the *thickvein* (*tkv*) or *saxophone* (*sax*) genes, which both encode cell surface receptors, render cells unresponsive to Dpp signaling (Massague 1996). Cells within clones that span the furrow and lack either of these receptors maintain high CycA, CycB, and CycE protein levels and also show inappropriate entrance into S phase (Penton et al. 1997; Horsfield et al. 1998). In contrast, ectopic expression of *dpp* in cells ahead of the furrow leads to a transient and reversible reduction in the number of cells entering S phase (Horsfield et al. 1998). These results implicate Dpp in the G1 arrest of cells within the furrow. Paradoxically, *dpp* is also required for growth of the early eye disc, a task that would seemingly be at odds with its role in preventing cells from making the G1/S transition. This apparent contradiction was resolved by the demonstration that Dpp forms a gradient in the eye and that a threshold level of Dpp protein is required to induce cell cycle arrest with the furrow (Firth et al. 2010).

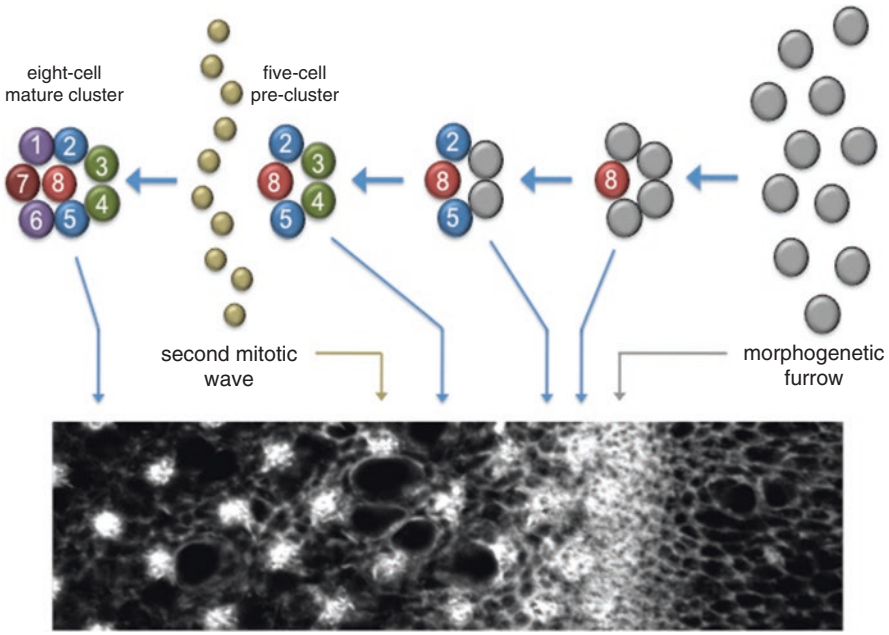

Fig. 8 The morphogenetic furrow, the second mitotic wave, and ommatidial assembly. As cells exit the furrow, a subset will exit the cell cycle and will adopt the fates of the first five photoreceptor clusters. All remaining cells will undergo a single round of mitosis and then adopt the fates of the last three photoreceptors as well as the cone and pigment cells. The schematic drawing marks the position of various events with the confocal image of the developing eye disc. Anterior is to the right in all images

Synchronization of the cell cycle within the furrow also requires *homothorax* (*hth*), which encodes a homeodomain containing transcription factor. Hth is a member of the retinal determination network, is expressed in the most anterior regions of the eye disc and is part of a biochemical complex that also contains the zinc finger transcription factor Teashirt (Tsh) and Ey (Bessa et al. 2002). The Ey-Tsh-Hth complex is required to repress the transcription of several other retinal determination genes such as *sine oculis* (*so*), *eyes absent* (*eya*), and *dachshund* (*dac*), thereby allowing cells in this zone to rapidly proliferate (Fig. 7). As cells begin to synchronize their cell cycles, *hth* expression is eliminated and *stg* expression is elevated. These mutually exclusive expression patterns hinted at potential regulation of *stg* by *hth*. Indeed, ectopic expression of *hth* within the eye leads to repression of *stg* transcription, maintenance of CycB levels, and a release from G1 arrest (Lopes and Casares 2009; Peng et al. 2009). These results suggest that, in order for cells to properly complete their last mitosis and then arrest in G1 within the furrow, *hth* expression must be repressed within the *stg* expression domain. How is the repression of *hth* transcription ahead of the furrow achieved? A prime candidate is Dpp signaling since the phenotypes associated with the loss of either *tkv* or *sax* mimic those that result from ectopic *hth* expression. Direct support of

this model comes from the observation that removal of either receptor (and thus a reduction in Dpp signaling) results in the maintenance of *hth* expression in cells within the furrow. Thus the current model is that long-range signaling by Dpp represses *hth* which in turns leads to the activation of *so*, *eya*, *dac,* and *stg* as well as the termination of *cycE*, *cycA,* and *cycB* (Fig. 7). These combined effects lead to cell cycle synchronization within the furrow.

Furrow Initiation and Progression

Birth of the Furrow

The eye disc is unusual in that, unlike all other imaginal discs, it is born without an established anterior-posterior (A/P) compartment boundary. Instead, the early eye disc contains just one of the two compartments; it is solely comprised of anterior tissue. Surprisingly, by the time that patterning of the eye is finished, the entire field has undergone a complete change in compartment identity. What used to be the anterior compartment is now the posterior compartment. This transition is mediated by the passage of the furrow across the eye disc and thus it represents a mobile compartment boundary (Fig. 2a). While differences in the use of mobile and stationary A/P boundaries exist, all imaginal discs use a common molecular mechanism to signal across and maintain compartment identities. Patterning of the embryo as well as the imaginal discs is dependent upon the activities of the *hh*, *dpp,* and *wg* genes. All encode secreted proteins with varying signaling ranges (Lee et al. 1992; Tabata and Kornberg 1994; Panganiban et al. 1990a, b; van den Heuvel et al. 1989; Gonzalez et al. 1991; Pfeiffer and Vincent 1999). *hh* is expressed in the posterior compartment and activates both *dpp* and *wg* expression in adjacent cells along the A/P compartment boundary (Basler and Struhl 1994; Capdevila et al. 1994). All three signaling pathways are present in the eye and play roles in both furrow initiation and progression (Fig. 9).

During the third and final instar, the morphogenetic furrow initiates at the posterior margin of the eye disc and begins its long journey across the eye primordium (Ready et al. 1976). Its initiation is restricted to a single point: the intersection of the posterior margin and the midline, which is called the posterior center (Tsai et al. 2007). Although the early eye lacks a posterior compartment and an A/P boundary, both *hh* and *dpp* are expressed along the posterior margin of the eye field prior to the initiation of the furrow. Just prior to furrow initiation, *hh* expression overlaps with the posterior center. *dpp*, on the other hand, while present along most of the posterior-lateral margins is distinctly absent from the posterior center (Fig. 9; Masucci et al. 1990; Blackman et al. 1991; Dominguez and Hafen 1997; Borod and Heberlein 1998). Loss of either gene inhibits initiation of the endogenous furrow while ectopic expression induces formation of ectopic furrows and neuronal differentiation within anterior quadrants of the eye field (Heberlein et al. 1995; Ma and

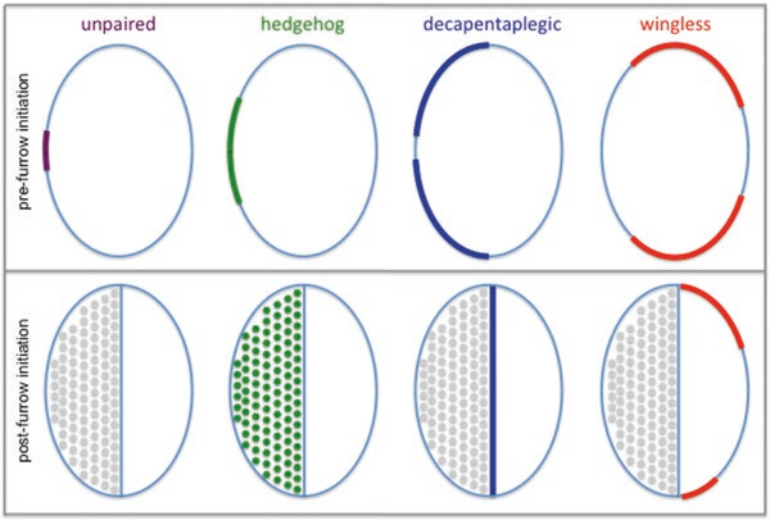

Fig. 9 Expression patterns of signaling molecules that regulate furrow and progression. The JAK/STAT, Hh, Dpp, and Wg signaling pathways are critical for regulating furrow initiation and progression. These schematics depict the expression patterns of the ligands for these pathways in early pre-furrow discs (upper row) and late post-furrow discs (bottom row). Anterior is to the right in all images

Moses 1995; Pan and Rubin 1995; Strutt et al. 1995; Wehrli and Tomlinson 1995; Wiersdorff et al. 1996; Dominguez and Hafen 1997; Pignoni and Zipursky 1997; Borod and Heberlein 1998). But despite the apparent requirement for both genes, Hh signaling alone is necessary and sufficient to initiate the furrow. This conclusion is based on the ability of Hh to induce neuronal differentiation in clones lacking *dpp* (Dominguez and Hafen 1997). What if any role does *dpp* then play in furrow initiation and why is it expressed at the posterior margin in the early eye? Interestingly, it may play a novel role in the maintenance of *hh* expression. Unlike the developing embryo and both wing and leg imaginal discs where Hh in the posterior compartment signals forward and activates *dpp* expression, in the eye there is a feedback loop between *hh* and *dpp*. Ectopic expression of *dpp* activates *hh* transcription, which is in turn required for *dpp*-induced neuronal differentiation. Thus the ability of *dpp* to initiate ectopic furrows and neural development is actually due to the initiation of *hh* transcription (Fig. 10; Pignoni and Zipursky 1997; Borod and Heberlein 1998).

The restriction of furrow initiation to the posterior center ensures that the retina is correctly patterned. Situations in which additional furrows are initiated at the anterior and/or lateral margins leave the eye disorganized and significantly smaller in size (Ma and Moses 1995; Treisman and Rubin 1995; Pignoni and Zipursky 1997). The Wg signaling pathway is tasked with limiting furrow initiation to the posterior center. *wg* is transcribed along the lateral margins of the disc and its loss results in the initiation of ectopic furrows (Figs. 9, 10; Ma and Moses 1995). Wg

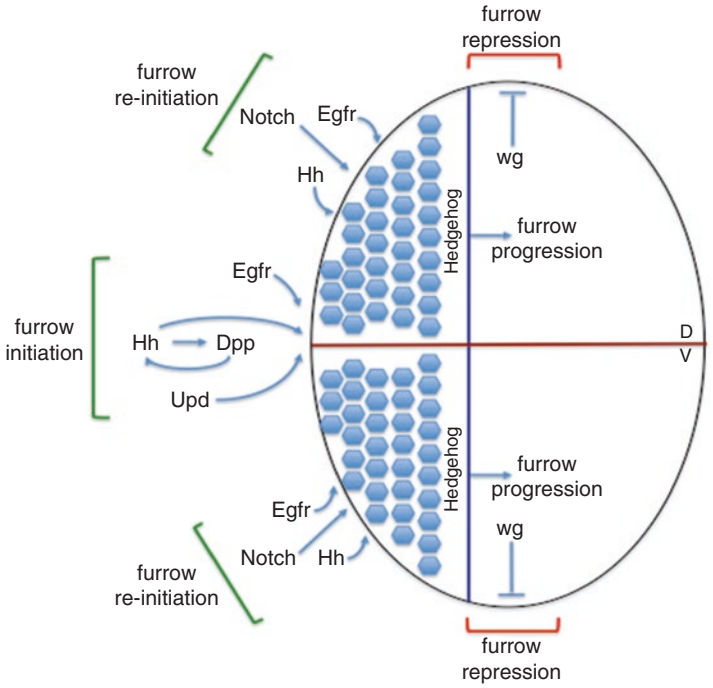

Fig. 10 Signaling pathways involved in birth, re-initiation, and progression. Schematic summarizing the position that the Hh, Dpp, Wg, Egfr, Notch, and JAK/STAT pathways occupy during the birth, re-initiation, and progression of the furrow across the eye disc. Note that Wg signaling is used to repress ectopic furrow initiation while all other pathways play roles in promoting the movement of the furrow. Anterior is to the right

signaling appears to be sufficient to block the furrow as ectopic expression of *wg* within the eye field inhibits progression of the endogenous furrow (Treisman and Rubin 1995). In addition to its role in furrow initiation, *wg* is also essential for delimiting the border between the compound eye and the surrounding head capsule (Royet and Finkelstein 1996, 1997).

In order for the furrow to initiate, *wg* expression must be repressed at the posterior center. A candidate for repressing *wg* transcription is the *unpaired* (*upd*) gene, which encodes a ligand for the JAK/STAT signaling pathway. In the developing eye, JAK/STAT signaling plays a major role in promoting cell proliferation (Bach et al. 2003; Chao et al. 2004; Tsai and Sun 2004; Ekas et al. 2006; Gutierrez-Avino et al. 2009). Prior to the initiation of the furrow, it is expressed exclusively at the posterior center (Fig. 9; Sun et al. 1995; Pignoni and Zipursky 1997; Tsai and Sun 2004). Reductions in *upd* expression lead to de-repression of *wg* along the posterior margin and a block in furrow initiation. Conversely, over-expression of *upd* along the lateral margins downregulates *wg* transcription and induces ectopic furrow initiation (Ekas et al. 2006; Tsai et al. 2007). Thus the combined activity of JAK/STAT and Hh signaling leads to the initiation of the furrow at the posterior center (with the Dpp

pathway functioning to maintain *hh* expression) while Wg activity prevents ectopic furrow initiation at the lateral margins (Fig. 10). Interestingly, as *wg* expression is relegated to the anterior-lateral margins of the disc, additional factors are likely used to prevent ectopic furrows from initiating at more posterior sections of the margins.

The initial birth of the furrow is followed by its continuous re-initiation along the posterior-lateral margins as each new column of ommatidia is added to the growing eye. An analysis of furrow initiation indicates that a critical control point precedes the birth of the furrow. The EGF receptor (Egfr) is required during this developmental window, as its inhibition completely blocks furrow initiation (Fig. 10; Kumar and Moses 2001a, b). It joins the Hh and JAK/STAT signaling pathways as being required for furrow birth (Heberlein et al. 1995; Ma and Moses 1995; Pan and Rubin 1995; Strutt et al. 1995; Wehrli and Tomlinson 1995; Dominguez and Hafen 1997; Ekas et al. 2006; Tsai et al. 2007). As there are roughly 32–34 ommatidial columns in a typical eye, the furrow re-initiates nearly three dozen times during the course of eye development. During the re-initiation process a second control point, also requiring Egfr signaling, was discovered. Further evidence implicated Notch signaling in furrow re-initiation as well. These two pathways, along with Hh signaling, are required to re-initiate the furrow along the posterior-lateral (Fig. 10; Wiersdorff et al. 1996; Kumar and Moses 2001a, b). The number of re-initiation control points is unknown but there is evidence that several may exist. The study that uncovered a role for Egfr signaling in furrow rebirth identified a control point for re-initiation as existing approximately twelve hours after the initiation of the furrow (Kumar and Moses 2001a, b). In a mutant allele of *hh*, one that contains a deletion of an eye-specific enhancer lying within the first intron (hh^{bar3}), the furrow fails to progress beyond the first 8–10 rows of ommatidia (Ives 1950; Mohler 1988; Heberlein et al. 1993; Lee et al. 1992; Ma et al. 1996; Pauli et al. 2005; Rogers et al. 2005). The resulting small eye is certainly due in large part to the reductions in *hh* levels within developing photoreceptor clusters. However, reductions in Hh signaling at the margins and a disruption in a re-initiation control point cannot be ruled out. Additional checkpoints may exist as the furrow stops short in several mutants such as $Drop^{1}$, $Wedge^{1}$, and ro^{Dom} (Heberlein et al. 1991, 1993; Tearle et al. 1994; Mozer 2001). As with hh^{bar3} mutants, it has been shown that a block in furrow progression is main underlying cause for the small eye phenotype of these mutants. Still, whether defects in furrow rebirth also contribute to the furrow stop phenotype remains to be determined.

Progression of the Furrow

Once the furrow has initiated and started to progress across the epithelium, the eye contains both anterior and posterior compartments as well as an A/P boundary. Like other tissues, *hh* is transcribed in the posterior compartment, which in the eye lies behind the morphogenetic furrow and is comprised of developing photoreceptor clusters (Fig. 9; Heberlein et al. 1993; Ma et al. 1993). Hh signaling, emanating

from the photoreceptor neurons, is required for progression of the furrow as its loss leads to a furrow stop phenotype and a small eye (Fig. 10; Ives 1950; Mohler 1988; Heberlein et al. 1993; Ma et al. 1993). How is its expression in developing photoreceptors regulated? An analysis of the eye-specific enhancer that is deleted in the hh^{bar3} and hh^{fse} mutants is particularly informative. The retinal determination protein Sine Oculis (So) and the Ets transcription factor, which mediates Egfr signaling, both bind to this enhancer and are required for the activation of *hh* transcription (Pauli et al. 2005; Rogers et al. 2005).

Similar regulatory mechanisms that exist between *hh* and *dpp* in other developmental contexts are in place during furrow progression. Hh signaling, originating from the photoreceptors, activates transcription of *dpp* (Masucci et al. 1990; Blackman et al. 1991; Heberlein et al. 1993; Borod and Heberlein 1998; Greenwood and Struhl, 1999). However, mutations that interfere with Dpp signaling result in only a small retardation in furrow progression, thus the major role of *dpp* in the furrow is to coordinate the synchronization of the cell cycle of cells anterior to the furrow (Burke and Basler 1996; Wiersdorff et al. 1996; Horsfield et al. 1998; Greenwood and Struhl 1999; Curtiss and Mlodzik 2000; Firth et al. 2010). The atypical relationship that leads to Dpp activation of *hh* at the margin during furrow activation does not appear to exist in the eye field proper during furrow progression.

Over the years several studies have provided differing accounts of how quickly the furrow traverses the eye disc. One study has documented the furrow laying down a new column of ommatidia approximately every 2 h (Campos-Ortega and Hofbauer 1977). Another report has clocked the furrow building an ommatidial column every 70 min in the posterior half of the eye but then slowing down to one hundred minutes in the anterior half (Basler and Hafen 1989). There are also suggestions that the furrow moves much more dynamically, alternating between periods of accelerations and decelerations (C. Spratford and J. Kumar, unpublished data). It will be important to definitively determine which rate is correct in order for an accurate understanding of how pattern formation and cell proliferation are coordinated in the eye. If the former outpaces the latter, as it happens when Wg signaling is blocked at the margins, the resulting eye will be small and disorganized (Ma and Moses 1995; Treisman and Rubin 1995). Thus it is important for the furrow to travel across the eye disc at a rate that allows for the eye to generate the requisite number of cells needed to generate approximately 800 ommatidia. This rate is likely to be influenced by several factors including the overall developmental timing and the physical dimensions of the eye disc.

Putting a Brake on Furrow Progression

As we have seen, mutations in several signaling pathways can either block the furrow from initiating or from progressing across the eye field. There are also many instances in which the furrow will slow without stopping (Strutt and Mlodzik 1997; Zelhof et al. 1997; Brennan et al. 1998; Dominguez 1999; Greenwood and Struhl 1999).

However, there are very mutations that result in the opposite phenotype, namely the acceleration of the furrow. Two genes that do appear to be involved in slowing the rate of furrow progression are *hairy* (*h*) and *extramacrochaetae* (*emc*). *h* encodes a basic helix-loop-helix (bHLH) DNA binding protein while *emc* encodes a helix-loop-helix (HLH) transcription factor that regulates transcription, not by binding to DNA, but by interacting with other bHLH proteins and sequestering them away from target enhancer/promoter sequences (Rushlow et al. 1989; Ellis et al. 1990; Garrell and Modolell 1990; Van Doren et al. 1991). Both proteins are enriched within a stripe of cells ahead of the morphogenetic furrow. Individual loss of either gene has no affect on furrow progression (Brown et al. 1991, 1995; Bhattacharya and Baker 2012). But surprisingly, the combined reduction in both proteins leads to an advancement in the furrow through mutant tissue (Brown et al. 1995). This led to the conclusion that both genes were simultaneously required to slow the furrow. However, in this experiment levels of *emc* were just reduced and not eliminated. In contrast, when *emc* is completely eliminated, the furrow will accelerate without the need for alterations in *h* expression (Bhattacharya and Baker 2009). It appears that the emc controls the rate at which the furrow progresses by regulating the levels of Ci^{ACT} (C. Spratford and J. Kumar, unpublished data).

The Furrow and Ommatidial Assembly

The first cell to be specified within each developing ommatidial cluster is the R8 photoreceptor (Ready et al. 1976; Tomlinson and Ready 1987; reviewed in Frankfort and Mardon 2002). This neuron is considered the founder cell and its initial determination (but not complete differentiation) is required for the correct recruitment and specification of subsequent photoreceptors (Frankfort et al. 2001). The decision as to which cell within each cluster will become the R8 neuron begins deep within the furrow and depends upon *atonal* (*ato*), which encodes a bHLH transcription factor (Fig. 11: Jarman et al. 1994, 1995; Dokucu et al. 1996). Ato protein is found within a stripe of cells at the entire edge of the furrow. Well within the furrow, *ato* expression is retained in periodically spaced clusters of approximately 10–12 cells that are called intermediate groups. Bridges consisting of 3–4 *ato* positive cells connect intermediate groups to each other. As cells exit the furrow, the number of *ato* positive cells per cluster is reduced to 2–3 and is now referred to as the R8 equivalence group. From this cluster, a single cell retains *ato* expression and is selected to adopt the R8 cell fate. The pruning of *ato* expression is dependent upon a number of inputs including Notch signaling and the transcription factors Rough and Senseless (Fig. 11; Cagan and Ready 1989b; Baker et al. 1996; Dokucu et al. 1996; Chanut et al. 2000; Frankfort et al. 2001; Pepple et al. 2008). The R8 will then activate the Egfr signaling pathway in two neighboring cells inducing them to adopt the R2/5 cell fate (Freeman 1994, 1996; Tio et al. 1994; Tio and Moses 1997; Kumar et al. 1998). Egfr signaling is used reiteratively to recruit the remaining cell

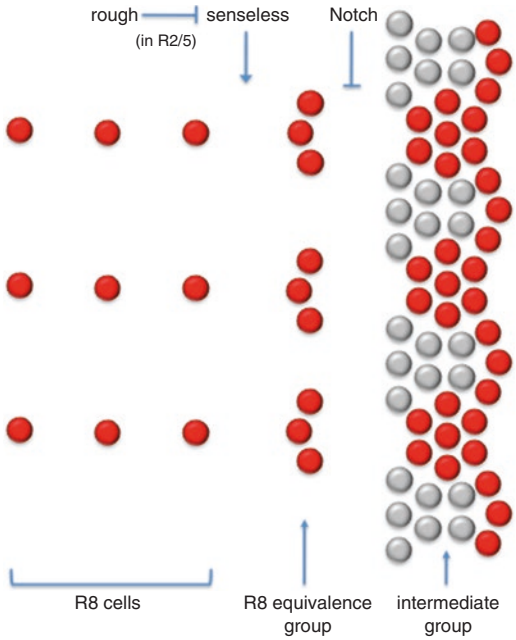

Fig. 11 R8 Specification begins in the morphogenetic furrow. The atonal bHLH transcription factor is required for the specification of the R8 founder cell. Its expression goes through successive waves of refinement until it is found within a single cell in each ommatidium. These pruning steps are mediated by the Notch pathway as well as two transcription factors Rough and Senseless. Loss of *ato* leads to the complete disruption in ommatidial assembly while over-expression of *ato* leads to ommatidia containing multiple R8 cells. Anterior is to the right

types, thus from this point onwards ommatidial assembly becomes a self-sustaining processes and the furrow no longer plays a role in cell fate specification (Freeman 1996, 1997).

The loss of *ato* expression in the eye disc results in a no-eye phenotype that is characterized by the complete elimination of photoreceptor, cone, and pigment cell development (Jarman et al. 1994). However, despite the lack of photoreceptor development, the morphogenetic furrow still initiates and progresses a considerable distance across the eye field (Jarman et al. 1995). It is not entirely clear how this occurs but presumably the levels of *hh* transcription at the margins are sufficient to initiate and propel the furrow. Interestingly, mutations in several retinal determination genes (*ey*, *so*, *eya*, *dac*) are associated with no-eye phenotypes but in contrast to *ato* mutants the furrow fails to initiate in these instances (Bonini et al. 1993; Cheyette et al. 1994; Quiring et al. 1994; Serikaku and O'Tousa 1994). These differences are likely to be attributed to the fact that at least three retinal determination genes regulate the expression of either *hh* and/or *dpp* (Hazelett et al. 1998; Pauli et al. 2005). The loss of either signaling pathway is amplified since both genes are in turn required for the proper functioning of the retinal determination network itself (Chen et al. 1999; Kango-Singh et al. 2003).

Concluding Remarks

Patterning of the *Drosophila* compound eye by the morphogenetic furrow has fascinated biologists for decades. This review is an attempt to briefly summarize our current knowledge of the mechanisms that underlie its movement across the eye field and some of its cellular characteristics. Despite the intense scrutiny that has surrounded the furrow, a number of outstanding questions continue to exist and are certainly worthy of future investigations. Some of the issues (just to name a few) that immediately come to mind include (1) discovering the identity of the timing mechanisms that govern the initiation of the furrow; (2) elucidating the means by which ectopic furrows are prevented from initiating outside of the *wg* expression domain; (3) determining the link between the rates of pattern formation and cellular proliferation; and (4) establishing the position of the control points that regulate furrow re-initiation and progression. These are just some of the questions that hopefully will be answered by the time the next review on the morphogenetic furrow is written.

Acknowledgement I would like to thank everyone who has contributed to our understanding of the mechanisms that regulate the initiation and progression of the morphogenetic furrow. I would like to also apologize to anyone whose work is not cited here. This work was supported by a grant from the National Eye Institute (R01 EY4863) to Justin P. Kumar.

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Ghost in the Machine: The Peripodial Epithelium

Brandon P. Weasner, Bonnie M. Weasner, and Justin P. Kumar

Introduction

The pair of eye-antennal discs of the fruit fly, *Drosophila melanogaster*, give rise to nearly all structures of the adult head (Fig. 1). It is often been described as being a monolayer epithelium consisting of a single sheet of columnar cells. But in point of fact, the eye-antennal disc is actually a closed sac consisting of two major layers: the disc proper (DP) and the peripodial epithelium (PE) (Fig. 2). The former is the columnar epithelium that most reports have focused on while the latter is a much less studied single layer of squamous cells that overlies the DP. The two epithelia are joined to each other along the edges by a thin layer of cuboidal cells. Overall, the structure of the eye-antennal disc resembles a pillowcase that has been stitched closed. During development, the PE makes significant contributions to the formation of the adult head. First, it produces multiple populations of head epidermal cells and several bristle types. Second, it influences the growth, specification, and patterning of the DP by transporting several signaling morphogens through translumenal cellular extensions. Lastly, it mediates the curling, fusion, and eversion of the two eye-antennal discs during pupal development. Despite these important functions, the PE has been overlooked for much of the last hundred years in which the *Drosophila* eye has been used as an experimental model system. It is often torn away so that "more interesting" features of the DP can be examined. As a result very little is known about the PE itself, how it contributes to the adult head, and the means by which it communicates with the DP. In contrast, studies of the DP abound and our knowledge of its development are vast. Thus, the PE serves as a striking

B. P. Weasner · B. M. Weasner · J. P. Kumar (✉)
Department of Biology, Indiana University, Bloomington, IN, USA
e-mail: jkumar@indiana.edu

A. Singh, M. Kango-Singh (eds.), *Molecular Genetics of Axial Patterning, Growth and Disease in Drosophila Eye*,
https://doi.org/10.1007/978-3-030-42246-2_4

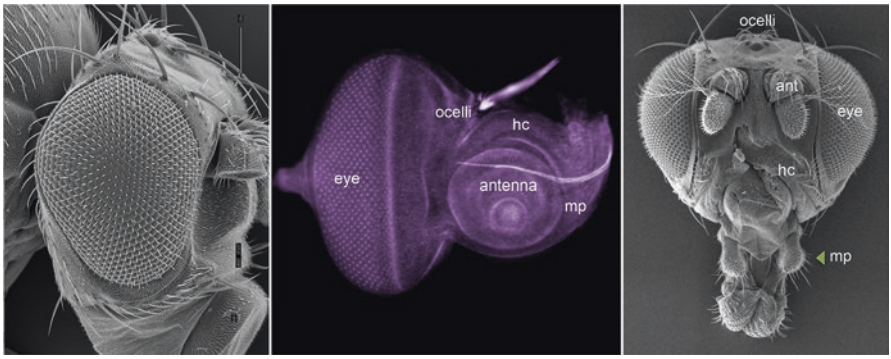

Fig. 1 The pair of the eye-antennal discs give rise to the adult head. (**a**, **c**) Scanning electron micrographs of adult heads. (**b**) Light microscope image of a third instar eye-antennal disc. *hc* head capsule, *mp* maxillary palps, *ant* antenna

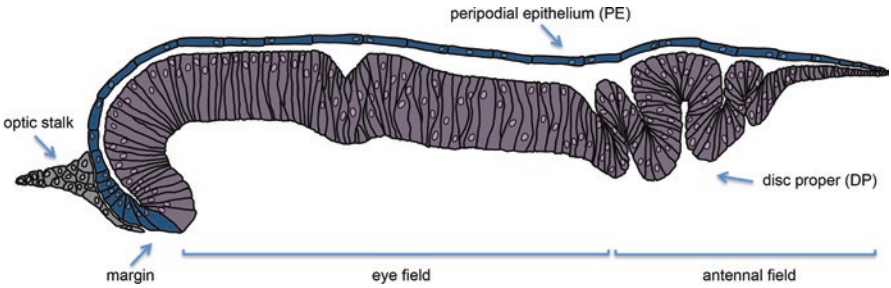

Fig. 2 This schematic depicts a cross section of an eye-antennal disc that shows the multiple cell types and layers of the eye-antennal disc. PE is in blue, DP is in purple, optic stalk is in grey. This drawing is a modification of a schematic in Fig. 3 of Gibson and Schubiger, 2001

example of Gilbert Ryle's concept of the Ghost in the Machine. In this chapter, we will summarize what is currently known about the PE and provide our view on where future studies might be directed.

Terminology: Epithelium Versus Membrane

Most studies of the PE refer to it incorrectly as a membrane despite the fact it has been known for 90 years to contain both cells and nuclei (Chen 1929; Pilkington 1942). Some articles place the word membrane in quotes, thus tacitly admitting that the term is being used inaccurately. In this chapter, it will be referred to as an epithelium, which is the anatomically correct term.

Morphology and Cell Types of the Eye-Antennal Disc

As described within the introduction, the eye-antennal disc is comprised of three cell layers that together form a sac-like structure—the DP, the PE, and the margin (Fig. 2). During the first two larval instars, the PE and DP are in contact with each other. The lumen forms during the first few hours of the third larval instar (Auerbach 1936). This organization is a common feature of all imaginal discs. Each layer contains a unique epithelial cell type with the DP consisting of columnar cells, the PE comprising of squamous cells, and the margin being made of cuboidal cells (Fig. 3). The PE lies atop the DP and a strip of margin cells joins the two sheets to each other (Figs. 2 and 4). Since the DP and PE are comprised of squamous and columnar cells

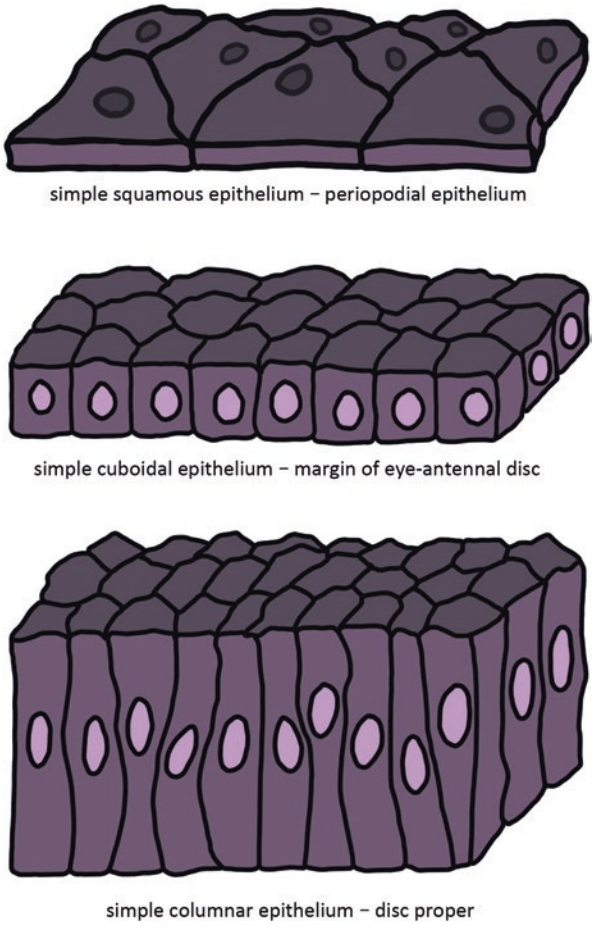

Fig. 3 This schematic depicts the three cell types that are present within the eye-antennal disc. The DP is composed of a layer of simple columnar cells, while the PE is comprised of a simple layer of squamous cells. The margin is made up of cuboidal shaped cells

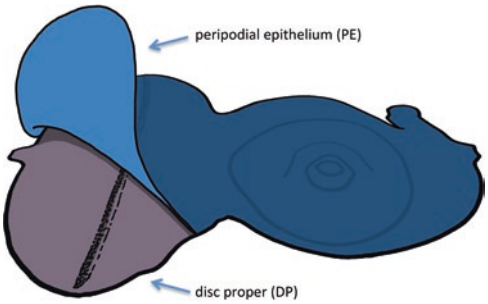

Fig. 4 This schematic depicts a third instar eye-antennal disc in which the PE has been partially "peeled away" in order to see the underlying DP

respectively but still have identical surface areas, the number of cells within the PE is significantly smaller than that of the DP (McClure and Schubiger 2005). The differences in cell numbers are also accompanied by distinct division patterns and cell doubling times (Adler and MacQueen 1984; Mathi and Larsen 1988; McClure and Schubiger 2005).

Little is known about how cells within each layer acquire their shape but one study of the eye-antennal disc suggests that the zinc finger transcription factor, Teashirt (Tsh), a member of the retinal determination (RD) network, is partly responsible for ensuring that cells of the DP retain their columnar shape (Bessa and Casares 2005). In the wing disc, Epidermal Growth Factor Receptor (EGFR) and Wingless (Wg) pathways are required to maintain the columnar shape of the DP. Forced activation of either pathway in the PE converts the squamous cells into columnar cells (Baena-Lopez et al. 2003). It remains an open question if the shape and fate of the cells in these layers can be separated. There is some evidence that these are separable features. Cells of the PE, which are normally squamous, will adopt a columnar shape in the early stages of pupal development (Milner et al. 1983)—this is required to contract the PE and curl the DP (see below).

The DP and PE Fate Choices

Within the imaginal disc, the DP and PE represent distinct cell fate choices and mechanisms are in place to ensure that cells of one layer do not adopt the fate of the other layer. For instance, the Scalloped (Sd)-Yorkie (Yki)-Homothorax (Hth) complex (Sd-Yki-Hth) maintains the fate of the PE within the eye-antennal disc. This complex is most often associated with the default promotion of growth within the DP of all imaginal discs. However, in the context of the PE of the eye-antennal disc, the Sd-Yki-Hth complex is tasked with maintaining the fate of that epithelial layer. Disruption of this complex forces the PE to adopt the fate of the DP, resulting in the formation of an ectopic eye within the PE (Zhang et al. 2011). An odd-skipped fam-

ily member, brother of odd with entrails limited (Bowl), controls the fate of the PE of the wing imaginal disc. Similar to the transformation described above, a reduction in Bowl levels within wing PE transforms it into an ectopic wing (Nusinow et al. 2008).

The Embryonic Origins of the Eye-Antennal Disc

Although the larval eye-antennal disc is a single physical unit, it is actually derived from several distinct embryonic cell populations. The first inkling that this is the case came from physical comparisons of the adult *Drosophila* head with that of other insects which indicated many structural similarities (Crampton 1942; Ferris 1950; Snodgrass 1935). Based on what was known at the time about arthropod head development, Ferris proposed that the *Drosophila* head (which is derived from the two eye-antennal discs) is composed of cells from six different embryonic head segments (Ferris 1950). Studies of gynandromorph heads and mosaic clones of body pigment and bristle mutants did indeed confirm that different portions of the eye-antennal disc do indeed develop from distinct populations of embryonic blastoderm cells (Baker 1978; Becker 1957; Haynie and Bryant 1986; Morata and Lawrence 1979; Ouweneel 1970; Struhl 1981; Vogt 1946; Wieschaus and Gehring 1976). However, all of these studies concluded that Ferris was incorrect in his assumption about cells coming from multiple embryonic head segments. Instead, the authors of these reports all proposed that the fly head was actually derived from a single segment.

This discrepancy was ultimately resolved when molecular markers and physical landmarks of developing embryos were used to show that the PE develops from the posterior-most regions of the dorsal pouch while the DP is comprised of cells from each of the three gnathal (mandible, maxillary, labium) segments, the anterior antennal segment, and the dorsal head (Jurgens and Hartenstein 1993; Younossi-Hartenstein et al. 1993). These widely spaced cell populations come together by a combination of cell migration and extensive programmed death of intervening cells. As the relevant cell populations make contact with each other, they sublime to form the eye-antennal disc, which is visible as a single unit for the first time at embryonic stage 15. By the end of embryogenesis, the entire eye-antennal disc is composed of approximately 70 cells (Madhaven and Schneiderman 1977).

Lineage tracings of the wing disc originally suggested that its PE, DP, and margin were all part of a single cell lineage. This was based on the finding that a single marked clone of cells could either span the PE and margin or the PE and DP (Pallavi and Shashidhara 2003). A later study showed, however, that some cells of that PE are displaced and invade the DP. This provided us with an alternate explanation for the earlier results with mosaic clones and suggested that the PE and DP of the wing are originally derived from two different lineages (McClure and Schubiger 2005). Lineage tracings of the eye-antennal disc confirmed the conclusions of earlier studies that the PE has a different embryological origin than the DP. It was also shown

that, like the wing disc, the margin of the eye-antennal disc is fated post-embryonically and is derived from the PE (Lim and Choi 2004).

The PE Gives Rise to Portions of the Adult Head

The first suggestion that the PE contains positional information and directly contributes cells to the adult head came from a morphological study of the butterfly, *Pieris brassicae*. Portions of the head epidermis (also called head capsule) were shown to originate not from the DP but rather from the PE (Eassa 1953). A similar examination of the developing blowfly, *Calliphora erythrocephala*, likewise demonstrated that that cells residing along the midline of the dorsal thorax are derived from the PE of the two wing discs (Sprey and Oldenhave 1974). The midline is where the two wing discs make contact and fuse with each other during metamorphosis. Peter Bryant and Ilan Deak proposed that something similar happens during development of the *Drosophila* thorax. Their ideas on how the PE contributes to adult thorax formation were important to understanding how fragments of wing imaginal discs could regenerate certain cell types, duplicate other structures, and in some cases transdetermine into completely different tissue types (Bryant 1975; Deak 1980). An analysis of wound healing within the wing disc using scanning electron microscopy showed that PE and DP cells along a fragment edge make contact (Reinhardt et al. 1977). The contact between cell layers is needed for short-range Hedgehog (Hh) signaling within the PE to induce regenerating anterior cells to transform into cells with posterior fate—this results in a duplication of several leg structures. However, if fragments lack a source of Hh then they will regenerate missing tissues without inducing fate conversions (Gibson and Schubiger 1999). Morphological studies of eye-antennal disc fusion (discussed later) and the generation of an eye-antennal disc fate map showed that portions of the adult head are, in fact, derived from the PE. This suggested that the original proposals of Bryant and Deak for the wing disc applied more generally and were also relevant to the eye-antennal disc.

The generation of a fate map for the eye-antennal disc made use of a disc transplantation technique that George Beadle and Boris Ephrussi pioneered in the 1930s. These authors dissected eye-antennal discs from larvae of eye color mutants and transplanted them into wild-type host larvae. The transplanted discs would undergo metamorphosis, along with the larval host, thereby allowing for the authors to recover the adult tissue and analyze the pigmentation pattern of the compound eyes that were derived from the transplanted discs. The authors would also transplant wild-type eye-antennal discs into eye color mutant hosts. These efforts were aimed at understanding if eye pigments were autonomous or non-autonomously functioning molecules (Beadle and Ephrussi 1935, 1936a, b, 1937; Ephrussi and Beadle 1937a, b).

In the 1960s, Ernst Hadorn modified this method and began transplanting fragments of imaginal discs into host larvae. He was interested in understanding how fragments would, on occasion, transdetermine and produce structures that would normally be derived from completely different imaginal discs (Hadorn 1968, 1978). John

Haynie and Peter Bryant used this method to generate a fate map of the eye-antennal disc. They fragmented eye-antennal discs and then transplanted individual pieces into third instar larval hosts. As the host larvae transitioned into pupae and then adults, the transplanted tissue, although fragmented, would still undergo metamorphosis and give rise to adult structures that were appropriate for their position within the eye-antennal disc. The adult tissues were scored for the presence of morphological features that were described by Ferris and each adult landmark was then assigned a position within the eye-antennal disc based on the disc fragment that gave rise to it. From this effort it was discovered that several structures including the occipital bristles, post-occipital sensilla, pre-mandibular bristles, and proximal rostrum sensilla, all of which are all found along the back of the adult head, actually develop from the PE (Haynie and Bryant 1986). It is worth noting that a portion of the proboscis (mouthparts) is also derived from the PE of the labial discs (Kumar et al. 1979).

Gene Expression in the PE and Their Roles in Adult Head Formation

The first genes to be ascribed roles in the PE were four members of the Antennapedia complex—*labial* (*lab*), *Deformed* (*Dfd*), *Sex combs reduced* (*Scr*), and *Antennapedia* (*Antp*). Each is expressed within domains of the PE that were determined by Haynie and Bryant to give rise to the occipital bristles, post-occipital sensilla, pre-mandibular bristles, and proximal rostrum sensilla. These structures are lost in select, viable, loss-of-function mutant alleles of each gene (Abzhanov et al. 2001; Chouinard and Kaufman 1991; Diederich et al. 1991; Jorgensen and Garber 1987). In addition, the maxillary palp, a structure that is not thought to arise from the PE, is also lost in *lab* and *Dfd* mutants suggesting that the PE must contribute to these and possibly to other head tissues (Chouinard and Kaufman 1991; Diederich et al. 1991; Merrill et al. 1987, 1989). Since these four Hox genes are only expressed in the PE and not within DP (Chadwick et al. 1990; Martinez-Arias et al. 1987; Wirz et al. 1986), it supports the idea that at least some PE cells give rise to portions of the adult head.

Interestingly, the absence of the maxillary palps and gena is also observed in a mutant allele of *decapentaplegic* ($dpp^{s\text{-}hc1}$) (Segal and Gelbart 1985; Spencer et al. 1982). *dpp* encodes a member of the Transforming Growth Factor β (TGFβ) superfamily and is expressed along the anterior-posterior (A/P) axis of the DP within all imaginal discs (Blackman et al. 1991; Masucci et al. 1990). Within the DP of the eye-antennal disc, it is required for the initiation and progression of the morphogenetic furrow as well as for correct patterning of the antennal field (Diaz-Benjumea et al. 1994; Heberlein et al. 1993; Ma et al. 1993; Theisen et al. 1996). The loss of the maxillary palps and the gena was somewhat surprising since the *dpp* expressing areas of the DP are not predicted to give rise to either of these two structures (Haynie and Bryant 1986). Since the aforementioned Hox genes are all expressed within the PE, it is possible that *dpp* would also be expressed there as well.

Indeed, *dpp* and *lab* are expressed in identical patterns along the ventral surface of the PE and their mutant phenotypes suggest that this region of the PE contributes to the development of the gena and maxillary palps (Stultz et al. 2005, 2006, 2012). The loss of *dpp* expression in the ventral PE is associated with an increase in Jun kinase (JNK) signaling and an induction of apoptosis—this further supports a model in which these cells also contribute to the formation of the maxillary palps and gena (Hursh et al. 2016; Park et al. 2015). We note that additional structures including the vibrissae and rostral membrane are also lost when *dpp* is removed from the PE (Park et al. 2015).

The enhancer that directs *dpp* expression within the PE is disrupted in the $dpp^{s\text{-}hc1}$ mutant allele. A scan of this enhancer identified binding sites for Lab and its co-factors, Extradenticle (Exd) and Hth. All three proteins physically bind to the enhancer. One of the Hth binding sites is deleted in the $dpp^{s\text{-}hc1}$ mutant. Enhancer activity is lost if the Lab, Exd, and Hth binding sites are altered. And *dpp* expression is reduced in *lab* and *hth* mutant clones (Stultz et al. 2012). The reduction, but not absence, of *dpp* expression suggests that this enhancer is regulated by additional factors. One such factor is the pair-rule gene, *odd paired* (*opa*). Loss of *opa* in the PE recapitulates the $dpp^{s\text{-}hc1}$ mutant phenotype and Opa is required for *dpp* expression in the ventral PE (Lee et al. 2007; Sen et al. 2010). Left unanswered by these studies is whether the *dpp* and *lab* expressing cells within the PE directly give rise to the maxillary palps and gena within the adult head or whether Dpp is signaling from this region to other cells within the PE or across the lumen to cells within the DP.

The PE Is Required for Curling, Fusion, and Eversion of the Eye-Antennal Disc

During larval development, the PE and DP both face the inside of the lumen. However, during the early phase of pupal development the discs undergo the process of eversion, so that both tissues eventually become part of the external surface of the adult. Eversion of the eye-antennal disc occurs once the morphogenetic furrow has finished generating the approximately 32–34 rows of unit eyes or ommatidia that comprise the adult compound eye. The first 26 or so rows are produced during the third larval instar while the final 6–8 rows are laid down during the first few hours of pupal development (Wolff and Ready 1993). Once the furrow reaches the eye/antennal border, the first step in disc eversion takes place—the eye field folds over the antennal field (the "curling stage"), so that the developing retina is in close proximity to the telescoping antennal field (Fig. 5). The curling of the eye-antennal disc is made possible by a drastic reduction in the area of the PE, which is now only about one-third of its maximum size. A contracting PE is essential for eye-antennal disc curling since cutting the taut PE reverts the eye-antennal disc to its initial linear shape (Milner et al. 1983). The decrease in the size of the PE is caused by cell shape changes. As the disc curls, cells of the PE go from being squamous (flat and irregu-

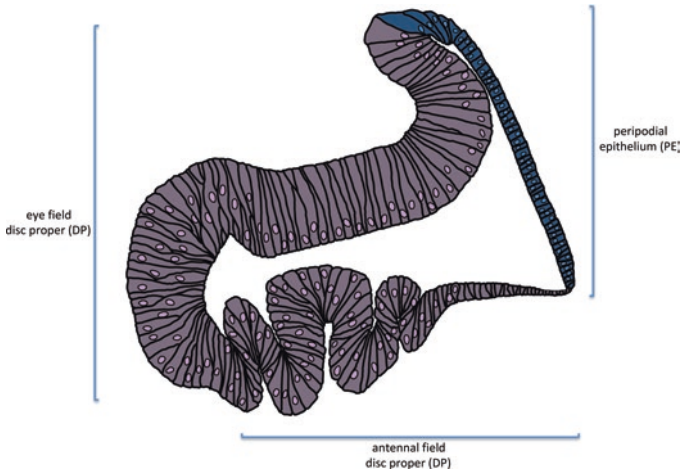

Fig. 5 This schematic depicts an eye-antennal disc that is undergoing the curling process during the early stages of pupal development. Curling of the disc takes place as a consequence of the PE cells changing shape, so that they are no longer squamous and instead have taken on a columnar shape. This leads to a contraction of the PE, which results in the pulling of the eye disc over the antennal segments

lar shaped) to being columnar (tall and thin) (Milner et al. 1983). This process is not unusual as similar shape changes are seen within the wing PE of the moth, *Manduca sexta* (Nardi et al. 1987).

As the two eye-antennal discs are curling, they are also fusing with each other to form a single intact head (Milner et al. 1984b; Milner and Haynie 1979). Early studies of gynandromorph heads had identified instances in which half of the head was of a male genotype while the other half was female (marked by epidermal and eye pigment color). In these cases, the lateral ocelli were of either one sex or another while the medial ocellus was a mixture of both sexes. Based on these results, it was postulated that each eye-antennal disc gives rise to one half of the adult head and that the suture formed by the fusion of the two discs runs through the medial ocellus and between the two lateral ocelli (Sturtevant 1929). Subsequent studies showed that if a single eye-antennal disc was transplanted into a host larva, then the adult head tissue that was derived from that disc contained a single lateral ocellus and a deformed medial ocellus. Similarly, if one of the two eye-antennal discs is first extirpated prior to the larva undergoing metamorphosis then one side of the adult head would be perfectly formed while the other half was completely missing. In these cases, one complete lateral ocellus and one small medial ocellus would remain. The transplantation and extirpation of eye-antennal discs confirmed that the two eye-antennal discs contribute equally to the formation of the adult head (Birmingham 1942).

During disc fusion, the eye-antennal discs contact each other along their respective medial edges (Fig. 6). Once initial contact is made, then both PE fuse with each other to form a single continuous sheet. Likewise, the two underlying columnar epithelia of the DP join together to form one large sheet as well (Milner et al. 1984b; Milner and Haynie 1979). The molecular mechanisms underlying the fusion of the two eye-antennal discs are not known. However, some hints as to what such mechanisms might look like can be gleaned from studies of wing imaginal disc fusion. In this instance, JNK signaling is required within the PE for the fusion of the wing discs—this fusion is necessary so that the two wing discs can form a single intact thorax. *puckered* (*puc*), which encodes a JNK-specific MAPK, is expressed specifically at the margins of the PE where the wing discs will normally fuse. Mutations that lower Puc levels block the fusion of the wing discs (Agnes et al. 1999; Zeitlinger and Bohmann 1999). It is not clear what happens to the two sets of cuboidal cells that once lined the medial edges of each eye-antennal disc and connected the medial edges of the PE and DP together. It is possible that they are subsumed into new single PE sheet. But it is just as likely that they are eliminated by programmed cell death.

Once the discs are both curled and fused together, then the entire head complex is turned inside out (eversion) through an opening at the posterior edge of the antennal discs (Fristrom and Frstrom 1993; Wolff and Ready 1993). This last step resembles the process by which a shirt or sweater is turned inside out. Many reports have

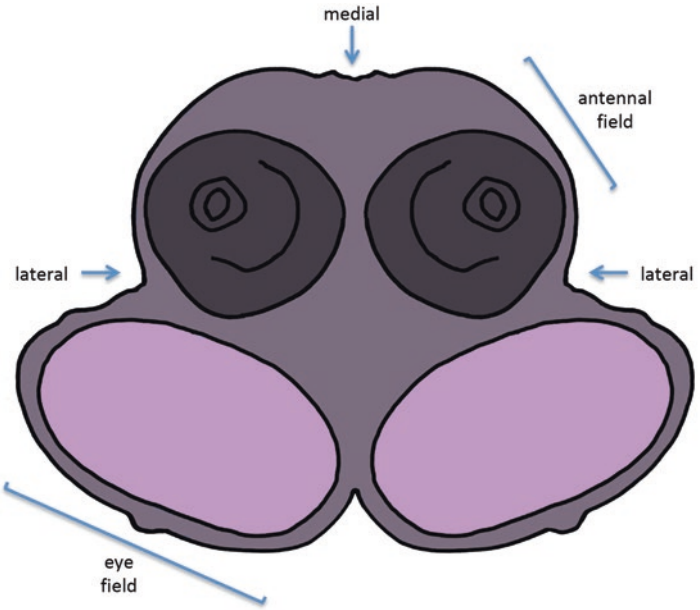

Fig. 6 This schematic depicts two eye-antennal discs that have undergone fusion along the medial edges during the early stages of pupal eye development. Please note that in this drawing, only the DP is shown. Also, we have not shown disc curling in this image

proposed that the wing and leg imaginal discs evert using similar inside out mechanisms (Milner 1977; Milner et al. 1984a; Poodry and Schneiderman 1970; Usui and Simpson 2000). However, the inside out model is not a universally accepted version of events. In fact, several studies have proposed a very different mechanism in which the disc proper actually pushes through a rupture in the middle of the peripodial epithelium (Auerbach 1936; Pastor-Pareja et al. 2004; Waddington 1941). A definitive conclusion has not been reached for the eye-antennal disc. This is due, in part, to the fact that pupal stage eye-antennal disc complexes are notoriously hard to culture for long periods of time and watching the process of disc eversion within the larva is technically challenging.

Signaling at the Margins of the Eye-Antennal Disc Regulates Major Aspects of Development

Like Dpp, the Hh, JAK/STAT, Wg, JNK, and EGFR signaling pathways play important roles within the developing eye. They control growth of the eye field, the shape of the retina, the specification of retinal fate, the establishment of dorsal and ventral compartments, the initiation and progression of the morphogenetic furrow, the establishment of planar cell polarity, and ommatidial assembly (Amore and Casares 2010; Dominguez and Casares 2005; Kumar 2011, 2013; Silver and Rebay 2005; Singh et al. 2012; Singh and Mlodzik 2012; Voas and Rebay 2004). Several of these roles take place at either the margin or within the PE. As we have discussed earlier, *dpp* expression within the ventral region of the PE is required for the formation of the maxillary palps and the gena (Stultz et al. 2005, 2006, 2012). It is also expressed along the posterior and lateral margins of the eye disc (Ma et al. 1993). Loss of *dpp* at the margin is associated with a failure of the furrow to initiate from and a consequent loss of pattern formation (Chanut and Heberlein 1997; Curtiss and Mlodzik 2000; Hazelett et al. 1998). Mutations in several members of the early acting (RD) network are characterized by severe reductions of the complete loss of retinal development (Kumar 2010). While differences exist amongst these mutants, a unifying theme is that *dpp* expression is lost (Baker et al. 2018; Hazelett et al. 1998; Mardon et al. 1994; Pignoni et al. 1997).

hh is expressed along the margin at the point where the optic stalk meets the posterior edge of the disc. Expression at this location, referred to as the "firing point," is essential for the birth of the furrow and the initiation of retinal patterning. Mutations that disrupt *hh* itself or its upstream regulators result in flies lacking the compound eyes (Bras-Pereira et al. 2006; Curtiss and Mlodzik 2000; Dominguez and Hafen 1997; Pauli et al. 2005). Unpaired (Upd), the ligand for the JAK/STAT pathway is also expressed at the firing point and its expression is critical for both growth and patterning of the eye field. Furrow initiation is blocked in JAK/STAT pathway mutants while ectopic signaling along the margins induces patterning formation (Chao et al. 2004; Ekas et al. 2006; Gutierrez-Avino et al. 2009; Tsai and

Sun 2004; Tsai et al. 2007). The JAK/STAT pathway is also required within the PE to repress *wg* expression and ensure that it is restricted to the dorsal and ventral margin (Ekas et al. 2006).

Wg signaling centers at the margins contribute to many aspects of eye development (Baker 1988; Legent and Treisman 2008). Early in development, Wg is required at and signals from the dorsal margin to establish the dorsal compartment (Heberlein et al. 1998; Maurel-Zaffran and Treisman 2000; Oros et al. 2010; Pereira et al. 2006). Later, Wg signaling from the margin is thought to contribute to the establishment of planar cell polarity (PCP) within the field of photoreceptor clusters behind the morphogenetic furrow (Lim and Choi 2004). The Wg pathway is also required to establish the boundary between the eye and head epidermis and to prevent pattern formation (via ectopic furrows) from initiating at the dorsal and ventral margins (Bras-Pereira et al. 2006; Ma and Moses 1995; Royet and Finkelstein 1996, 1997; Treisman and Rubin 1995). Lastly, during pupal development, Wg is required along the periphery of the compound eye to create and maintain a sharp eye-head border (Kumar et al. 2015; Lim and Tomlinson 2006; Tomlinson 2003).

The PE Sends Signals to the DP Directly Via Translumenal Extensions

In addition to their roles along the margins, several of these pathways are, in addition to Dpp, expressed broadly within the PE but function to control development of the entire eye-antennal disc (Atkins and Mardon 2009). For example, expression of the Notch pathway ligands Serrate (Ser) and Delta (Dl) in the PE is required for robust growth of both the PE and the DP. Reductions in Ser/Dl or alterations to their activities lead to small compound eyes (Gibson and Schubiger 2000; Kooh et al. 1993). Similarly, Hh is required within the PE for *Ser* expression within the disc proper. Disruption of *hh* transcription in the PE affects *Ser* dependent growth and PCP within the DP (Cho et al. 2000). Broad expression of signaling molecules within the PE is not unique to the eye-antennal disc. For instance, *hh* is expressed uniformly within the PE of the wing imaginal disc (Gibson and Schubiger 1999).

How does the PE communicate to and exert its effects on the DP during development? Morphologically, the apical surfaces of the PE and the DP face each other (Pallavi and Shashidhara 2005), therefore juxtacrine and paracrine signaling can easily occur. During the first two larval instars, the PE and DP are in direct contact with each other (Auerbach 1936). Such contact would allow for direct juxtacrine signaling between the two layers. However, during the third larval instar, the lumen of the eye-antennal disc forms (Auerbach 1936) which makes signaling via juxtacrine mechanisms all but impossible. Two possible mechanisms remain. The first would invoke secretion of ligands from the PE, diffusion through the lumen, and capture by cells within the DP. Such a mechanism is unlikely to work well as there would be little to no spatial control over the reception of the secreted signals and as

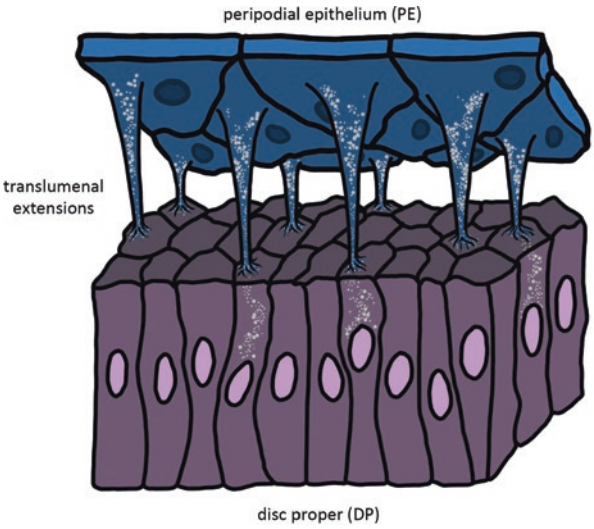

Fig. 7 This schematic depicts microtubule-based translumenal extensions that transport morphogens (grey) from the PE (blue) to the DP (purple) of the eye-antennal disc

we have seen above, the ligands in the PE have very specific effects on the development of the DP.

Kwang Choi and Gerold Schubiger described a second mechanism in which morphogens are not secreted into the lumen of the disc but are rather transported through the lumen via cellular appendages of PE cells called translumenal extensions (Fig. 7). These cellular structures are observed in developing eye-antennal, leg, and wing discs, which suggests that this is a common signaling mechanism (Cho et al. 2000; Gibson et al. 2002; Gibson and Schubiger 2000). Interestingly, the translumenal extensions that are associated with the PE of the eye-antennal disc appear to be signaling to cells within and behind the morphogenetic furrow. Disruption of these extensions or the molecules that are being transported within them abolish the mitotic waves that are associated with the furrow (Gibson et al. 2002). It is not clear if extensions connect portions of the PE with other parts of the DP.

Cellular extensions called cytonemes have also been reported to traffic signaling molecules in wing and eye-antennal discs (Fig. 8) (Ramirez-Weber and Kornberg 1999, 2000; Roy et al. 2011). While both cellular structures are tasked with moving morphogens, translumenal extensions and cytonemes do differ in several aspects. The major difference is that translumenal extensions project from one layer (the PE) through the lumen of the imaginal disc and contact the other layer (the DP) while cytonemes project from peripheral cells of the DP to signaling centers along the A/P axis of the DP itself (Figs. 5 and 6). Another significant difference is that cytonemes are actin-based structures while translumenal extensions are composed of microtubules. It is not clear why the differential use of the actin and microtubule cytoskeletons exist.

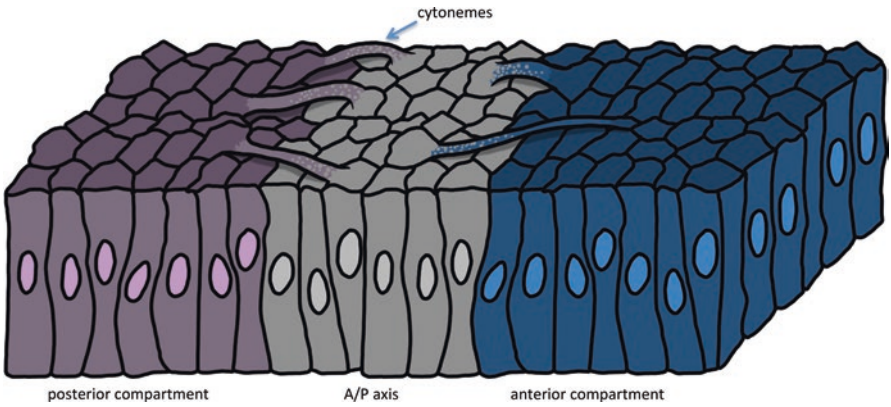

Fig. 8 This schematic depicts a small region of the DP of an imaginal disc. The cells in blue represent the anterior compartment, the cells in purple represent the posterior compartment, and the cells in grey represent the A/P axis. Cells from the anterior and posterior compartment extend actin-based cellular processes called cytonemes into the A/P boundary. Cells at the A/P border secrete morphogen-containing vesicles, which are captured by the cytonemes and transported back to individual cells

An interesting study by Lucy Firth and Nicholas Baker suggested that the DP also influences the development of the PE via transmission of signaling molecules. They showed that the expression of a secreted form of the EGFR ligand Spitz (sSpitz) within developing photoreceptor neurons induced significant gene expression changes within the PE (Firth and Baker 2007). In this case, sSpitz is probably diffusing across the lumen. However, it is not clear if Spitz or other morphogens are normally trafficked to the PE. If this is indeed the case, it will be interesting to determine if cells of the DP extend translumenal processes and drop morphogen-containing vesicles on the PE or if there is free diffusion of ligands across the lumen.

It makes sense that signals controlling patterning and growth would be trafficked in both directions. For example, despite the differences in cell numbers and shape, the two epithelial sheets must grow in proportion to each other. Therefore, one could imagine that the PE and DP keep track of each other's growth through back and forth transmission of growth signals. Such a requirement is likely to be true of wild-type and mutant discs since both tissues have the same surface area irrespective of whether both the PE and DP are wild type, both are mutant for an individual gene, or if one is wild type and the other is mutant. This was nicely shown by the elimination of the gap junction proteins Innexin2 or Innexin3 in both layers or selectively in one layer. In each instance, the PE and DP were reduced proportionally and the adults had small eyes (Richard et al. 2017; Richard and Hoch 2015).

Transcriptional Networks in the PE

As this review indicates, a lot of interest has centered on how the PE and DP communicate with each other during development. As a result, less attention has been paid to the transcriptional networks that reside and function within the PE itself. Such networks would be important for establishing the fate of the PE, for dividing the PE into dorsal and ventral compartments, for promoting its growth, and for specifying the fates of individual domains of the PE (for use within the adult head). As described above, the Sd-Yki-Hth complex controls basic PE fate and several Hox genes control some later cell fate choices. In addition to these factors, several members of the RD network such as Eyeless (Ey), Sine Oculis (So), Eyes Absent (Eya), and Eyegone (Eyg) as well as the fly homolog of Microphthalmia-associated transcription factor (Mitf) are present within the PE (Atkins and Mardon 2009; Gibson and Schubiger 2000; Halder et al. 1998; Hallsson et al. 2004). Expression of a dominant negative version of Mitf or an RNAi construct that targets Ey within the PE using the c311 GAL4 driver (Manseau et al. 1997) leads to retinal specification and patterning defects (Baker et al. 2018; Hallsson et al. 2004). The roles played by the other transcription factors in the PE are not yet known.

Future Directions

Although we have learned a lot about how the PE contributes to the development of imaginal discs, there still remains a lot to be learned. Compared to what is known about the imaginal disc DPs, our knowledge of the PEs is miniscule. This is in part because most of the studies cited herein were conducted many decades ago when there were very few molecular markers and genetic tools. It is also due to a failing of many of us to appreciate the contributions that the PE makes to the eye-antennal disc and the adult head. Figure 6B of Don Ready and Seymour Benzer's seminal paper on the developing eye illustrates this point (Ready et al. 1976). In this image, the PE is peeled away in order for the underlying disc and morphogenetic furrow to be viewed. While the removal of the PE was necessary to see the furrow, its extirpation stands as a metaphor for how most *Drosophila* eye researchers viewed the PE as something to be simply discarded, so that "more interesting" portions of the disc can be examined.

Our understanding of the role that the PE of each imaginal disc plays in development will be greatly enhanced by using modern lineage tracing systems to generate more sophisticated fate maps; high-throughput genomic methods to obtain temporal and spatial gene expression profiles; large RNAi and loss-of-function mutant collections to mutagenize the PE and assay the resultant effects on DP and adult development; super-resolution microscopy to view signaling between the different epithelial layers; and light sheet microscopy to visualize the cell movements of imaginal discs during metamorphosis. Such efforts will lead to a correction of a category mistake that Ryle warned us about.

Acknowledgments We first thank every researcher that has worked on the peripodial epithelia of imaginal discs over the last century—Peter Bryant, John Haynie, Martin Milner, and Gerold Schubiger deserve special mention. We would also like to thank Alison Ordway and Alison Smith for their comments on an earlier draft of this chapter. This work is supported by a grant from the National Eye Institute (R01 EY014863) and funds from the Department of Biology, the College of Arts and Sciences, and the Office of the Vice Provost for Research at Indiana University to Justin P. Kumar.

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Cell Polarity in *Drosophila* Retina

Sang-Chul Nam

Differentiation and Morphogenesis of *Drosophila* Retina

The compound eye of *Drosophila* is made up of about 800 ommatidia, each of which is comprised of a cluster of eight elongated columnar photoreceptor cells covered by a thin layer of pigment cells (Kumar and Ready 1995; Longley and Ready 1995). These clusters of 8 photoreceptor cells (R1–R8) are made in the eye disc epithelium during the third-instar larval stage, before photoreceptor morphogenesis takes place. At 37% pupal development (pd) stage, the apical region of each of the photoreceptor cells is involuted by 90°, reorienting the apical domains towards the center of the cluster (Fig. 1) (Kumar and Ready 1995; Longley and Ready 1995). At this time, the apical membrane domain, having been localized at the center of the photoreceptor cluster, is now surrounded immediately by adherens junction (AJs), followed by the basolateral domains (Fig. 1) (Izaddoost et al. 2002; Pellikka et al. 2002). The formation of the rhabdomere from the apical surface of the photoreceptor cells begins at 55% pd and involves a series of complex cell–cell signaling interactions and the rapid expansion of the plasma membrane (Kumar and Ready 1995; Longley and Ready 1995). Along the length of each ommatidial column extends a light sensitive, tightly packed array of 60,000 microvilli called a rhabdomere (Kumar and Ready 1995; Longley and Ready 1995). Because of the enormity of this growth/elongation and the rapidity with which it occurs, even small signaling defects can cause dramatic phenotypic consequences in the developing eye (Table 1).

S.-C. Nam (✉)
Department of Biology, Hillsdale College, Hillsdale, MI, USA
e-mail: snam@hillsdale.edu

A. Singh, M. Kango-Singh (eds.), *Molecular Genetics of Axial Patterning, Growth and Disease in Drosophila Eye*,
https://doi.org/10.1007/978-3-030-42246-2_5

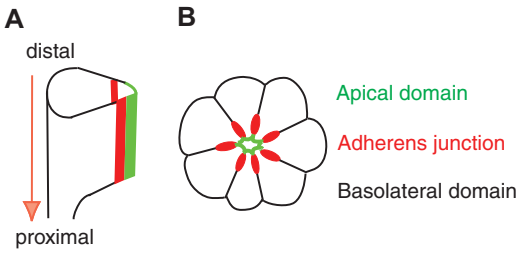

Fig. 1 Morphogenesis of *Drosophila* pupal photoreceptors. (**a**) Side view of developing photoreceptors at mid-stage of pupal development. The photoreceptors elongate from distal to proximal (arrow). (**b**) Cross section of mid-stage pupal photoreceptors. Apical domain (green) localizes apical to AJ (red) in the center of a photoreceptor cluster

Apicobasal Cell Polarity Genes

The establishment and maintenance of cell polarity is an essential feature of all eukaryotic cells and is critical for the integrity of the organism. Recent studies have begun to reveal the molecular and genetic basis of apical-basal cell polarity by identifying important proteins involved in cell polarity determination and junction formation (Bilder 2001a, b; Ohno 2001). Accumulating evidence suggests that important cues for the establishment of cell polarity are provided by the function of at least four evolutionarily conserved protein complexes. These are Crb complex of Crb/Sdt/Patj (Bachmann et al. 2001), Par complex of Par-6/aPKC/Par-3 (Ohno 2001), Scribble complex of Scrib/Dlg/Lgl (Bilder and Perrimon 2000), and Yurt complex of Yurt/Coracle/Neurexin-IV/Na-K-ATPase (Laprise et al. 2009). The Crb and Par complexes localize at the apical membrane domains or AJs, but the Scribble and Yurt complexes reside on the basolateral domains. Further, there is the fifth complex of Par-1/Lkb1(Par-4)/AMPK. All five cell polarity complexes contribute to establish, maintain, and regulate the cell polarity through synergic and antagonistic collaborations (Laprise and Tepass 2011; Tepass 2012; Tepass et al. 2001).

Crb/Sdt/Patj Complex in Retina Development

Crb complex including the Crb, Sdt, and Patj were discovered in *Drosophila* (Bhat et al. 1999; Tepass and Knust 1993). The *crb* and *sdt* genes were identified genetically as essential components for organizing apical-basal polarity and AJs in early embryonic epithelia (Bachmann et al. 2001; Bhat et al. 1999; Hong et al. 2001; Tepass et al. 1990). Genetic interaction studies suggested that *sdt* acts downstream of *crb* in the same pathway (Grawe et al. 1996; Tepass and Knust 1993). Molecular analysis of Crb and Sdt has shown that they are directly associated in the apical plasma membranes of epithelial cells (Bachmann et al. 2001; Hong et al. 2001). Crb is a transmembrane protein with a long extracellular domain and a short C-terminal

Table 1 Cell polarity genes and their regulators affecting retina morphogenesis

Drosophila	References	Vertebrate	References
abl	Xiong and Rebay (2011)		Nunes et al. (2001)
ampk/lkb1	Amin et al. (2009), Poels et al. (2012), Spasic et al. (2008)		Samuel et al. (2014), Xu et al. (2018)
apkc	Nam and Choi (2003), Nam et al. (2007)	*heart and soul*	Horne-Badovinac et al. (2001)
crb	Chartier et al. (2012), Izaddoost et al. (2002), Johnson et al. (2002), Nam and Choi (2003), Pellikka et al. (2002), Pocha et al. (2011)	*Crb1,Crb2*	den Hollander et al. (1999), Mehalow et al. (2003), Pellikka et al. (2002), Quinn et al. (2018a, b), Zou et al. (2012)
baz	Hong et al. (2003), Nam and Choi (2003), Nam et al. (2007), Walther and Pichaud (2010)	*Par-3, Pard3*	Sottocornola et al. (2010), Wei et al. (2004)
chaoptin	Zelhof et al. (2006)		
cnn	Chen et al. (2011)		
cofilin	Pham et al. (2008)		Kumar et al. (2016)
eyes shot	Husain et al. (2006), Zelhof et al. (2006)	*spacemaker*	Abd El-Aziz et al. (2008), Collin et al. (2008)
kinesin-1	League and Nam (2011)		Jiang et al. (2015)
kinesin-2	Mukhopadhyay et al. (2010)	*Kinesin-2 (KIF3)*	Avasthi et al. (2009), Jimeno et al. (2006a, b), Lewis et al. (2017, 2018), Lopes et al. (2010), Trivedi et al. (2012)
MRCK	Zihni et al. (2017)		
moesin	Karagiosis and Ready (2004)		
myosin V	Li et al. (2007), Pocha et al. (2011)	*myosin V*	Libby et al. (2004)
par-1/pp2a	Nam et al. (2007)		
par-6	Nam and Choi (2003), Nam et al. (2007)		
patj	Nam and Choi (2006), Richard et al. (2006), Zhou and Hong (2012)		
pi3k/pten	Pinal et al. (2006)		Jo et al. (2012), Sakagami et al. (2012)
prominin	Nie et al. (2012), Zelhof et al. (2006)		Nie et al. (2012)
rab11	Satoh et al. (2005), Wu et al. (2005)	*Rab11*	Ying et al. (2016)
sdt	Hong et al. (2003), Nam and Choi (2003), Nam et al. (2007)	*Pals1, nagie oko*	Cho et al. (2012), Park et al. (2011), van Rossum et al. (2006), Wei and Malicki (2002), Zou et al. (2008)
sec6, sec8	Beronja et al. (2005)		
shot	Mui et al. (2011)	*MACF1*	May-Simera et al. (2016)
spastin	Chen et al. (2010)		
spectrins	Chen et al. (2009)		Isayama et al. (1991), Papal et al. (2013)

(continued)

Table 1 (continued)

Drosophila	References	Vertebrate	References
tau	Bolkan and Kretzschmar (2014), Nam (2016)	*Tau*	Chiasseu et al. (2017), Chidlow et al. (2017), Ho et al. (2015), Mazzaro et al. (2016), Xu et al. (2015), Zhao et al. (2013)
yurt	Laprise et al. (2006)	*mosaic eyes*	Hsu et al. (2006), Jensen and Westerfield (2004)
wasp	Zelhof and Hardy (2004)	*wasp*	Singh et al. (2013)

cytoplasmic tail that recruits Sdt and Patj through direct biochemical interactions (Hong et al. 2001; Roh et al. 2002) and now it is treated as a single protein complex (Fig. 3).

The apical-basal polarity is prominent in the photoreceptors due to the photosensitive organ, rhabdomere, formed on the apical surface of the cell. During pupal eye development, the apical domain of differentiating photoreceptors undergoes dynamic reorganization of the cell shape and size, resulting in the formation of rhabdomeres (Kumar and Ready 1995; Longley and Ready 1995). Recent studies have shown that Crb plays important roles in morphogenesis of the photoreceptor rhabdomere, providing evidence that at least some proteins involved in the apical-basal polarity of embryonic epithelia have essential roles in the organization of photoreceptors (Izaddoost et al. 2002; Pellikka et al. 2002). Crb is specifically localized to the rhabdomere stalk, a membrane domain that is juxtaposed apically to the emerging rhabdomere and basally to the AJ. Crb is required for positioning and growth of rhabdomere and AJ during the critical period of photoreceptor extension along the proximal-distal axis of the retina. Further analysis of Crb function has shown that the intracellular domain is necessary for the recruitment of AJ as well as localization of rhabdomere stalk (Izaddoost et al. 2002).

Importantly, Crb's mammalian homolog localizes to the region corresponding to the rhabdomere stalk membrane, that is, the inner segment between the outer segment (analogous to the rhabdomere) and the AJ of rod photoreceptors (Pellikka et al. 2002). Furthermore, mutations in *CRB1*, one of Crb homologs in human, cause severe retinal dystrophies such as retinitis pigmentosa type 12 (RP12) (den Hollander et al. 1999) and Leber congenital amaurosis (LCA) (den Hollander et al. 1999). These studies suggest that Crb and other cell polarity components involved in the specification of apical membrane of photoreceptors might be evolutionarily conserved. Crb's mammalian homolog, Crb1, was found to be an essential role in retinal differentiation in mice (den Hollander et al. 1999; Mehalow et al. 2003) and in zebrafish (Zou et al. 2012). Crb is also associated with light-induced retinal degeneration (Johnson et al. 2002) and superoxide-dependent retinal degeneration (Chartier et al. 2012). The Crb's degeneration protection was found to link with rhodopsin 1 trafficking (Pocha et al. 2011) and Rac1-NADPH oxidase complex activity (Chartier et al. 2012).

Sdt also shows colocalization with Crb specifically to the rhabdomere stalk region of the photoreceptor in pupal retina (Hong et al. 2003; Nam and Choi 2003).

In the mutant of *sdt*, Crb and Patj were almost completely absent in rhabdomere stalks. In contrast, in the mutant of *crb*, Sdt was mislocalized together with Patj from the rhabdomere stalk (Nam and Choi 2003). These results provide an evidence of the inter-dependent function of Crb complex proteins in the developing retina. Sdt's vertebrate homolog, Pals1, was also reported to play important roles in retinal differentiation (Cho et al. 2012; Park et al. 2011) using a conditional knout-out technique. Previously, Sdt's zebrafish homolog, Nagie oko, was found to localize to the apical cell junctions of the retinal neuroepithelium and have an essential role in retinal differentiation (Wei and Malicki 2002; Zou et al. 2008).

Crb and Sdt are required for rhabdomere elongation and AJs during pupal photoreceptor morphogenesis (Hong et al. 2003; Izaddoost et al. 2002; Nam and Choi 2003; Pellikka et al. 2002). Patj binds Sdt to form a conserved heterotrimeric Crb complex (Roh et al. 2002). Recently, Patj's function in photoreceptor morphogenesis was clearly demonstrated. First, synthetic hypomorph of *patj* showed late-onset degeneration of photoreceptor cells in adult eye although the mutant eyes develop relatively normally (Nam and Choi 2006; Richard et al. 2006). Second, analysis of synthetic null mutant of *patj* null and $patj^{RNAi}$ demonstrated that Patj is essential for early development of the animal and for morphogenesis of AJ and apical membrane domains of photoreceptor cells during pupal development (Nam and Choi 2006). The role of patj in retina development was further demonstrated using a knock-out mutant of *patj* (Zhou and Hong 2012). In addition to Crb, Sdt and Patj were also associated with progressive light-induced retinal degeneration (Berger et al. 2007; Richard et al. 2006).

Par-3/Par-6/aPKC Complex in Cell Polarity

The Par3/Par6/aPKC complex is an evolutionarily conserved regulator of cell polarity that plays a central role in forming and maintaining cell junctions in early embryonic epithelial cells and in determining asymmetric cell division (Ohno 2001). The first discovery of Par-3 was done in the partitioning-defective phenotype which was manifested in the first embryo cell division in *C. elegans* (Kirby et al. 1990). The normal *C. elegans* embryo goes through the asymmetric cell division to generate the differential cell fate of the two daughter cells. But, the *partitioning-defective (par)* mutants show the symmetric cell division based on the loss of the polarized distribution of fate determinants. Based on this initial screening, several *par* mutant genes including *par-3* and *par-6* were isolated (Kirby et al. 1990). Later, through biochemical analysis Par-3, Par-6, and aPKC were found to bind each other directly through the protein–protein interaction (Fig. 3), therefore the single protein complex hypothesis was established (Macara 2004a, b). This single complex is called by a Par complex composed of Par-3(Baz)/Par-6/aPKC (Fig. 3). Baz contains three PDZ domains that can mediate protein–protein interactions with Par-6 (Petronczki and Knoblich 2001) and aPKC (Wodarz et al. 2000) and it is thought that these three proteins form a polarity complex (Macara 2004a, b) (Fig. 3).

The cross-talk between the Par complex and Crb complex was identified through the direct protein–protein interactions. The Sdt-Par-6 (Hurd et al. 2003), Crb-aPKC (Sotillos et al. 2004), Sdt-Baz (Krahn et al. 2010; Sotillos et al. 2004), or Patj-Par-6 (Nam and Choi 2006) binding causes the physical interaction between the Crb and Par complex, and this interaction affects the tight collaboration between these two complexes to generate cell polarity or organ morphogenesis (Hurd et al. 2003; Nam and Choi 2006; Sotillos et al. 2004).

Par-3(Baz)/Par-6/aPKC in Retina Development

Par-6/aPKC colocalize with Crb complex proteins in photoreceptor cells during pupal stages. Both Par-6 and aPKC colocalized with Crb/Sdt/Patj at the rhabdomere stalks. In contrast, Baz localized at the AJ of photoreceptors basal to the rhabdomere stalk (Hong et al. 2003; Nam and Choi 2003). In *baz*, *par-6* or *apkc* mutant photoreceptors, Crb/Sdt/Patj and AJ were strongly reduced and/or mislocalized (Hong et al. 2003; Nam and Choi 2003). These results demonstrate that Par-6/aPKC/Baz complex proteins are essential for proper localization of Crb complex and AJ components. In contrast, Par complex remained in the membrane although mislocalized, in the absence of Crb complex (Nam and Choi 2003). This data suggests that Par complex acts upstream to the Crb complex. The hierarchy among the Par complex of Baz/Par-6/aPKC was further examined and found that Baz acts upstream to Par-6/aPKC (Nam et al. 2007). Therefore, Baz is a nodal component for apical targeting of Par and Crb complexes (Nam et al. 2007).

The knowledge of Par complex in vertebrate eyes is very limited except the aPKC in zebrafish retina (Horne-Badovinac et al. 2001). The *heart and soul* mutation in aPKClambda (an ortholog of aPKC) caused AJ defects in zebrafish retina (Horne-Badovinac et al. 2001). The localization of Par-3 in mouse retina was examined (Sottocornola et al. 2010), but the functional role of Par-3 in vertebrate retina is not known yet.

Localization of Baz at AJs of Photoreceptors

Vertebrate Par-3, Baz homolog, localizes to the apical tight junction in vertebrate epithelial cells (Izumi et al. 1998; Suzuki et al. 2001). In most models, Baz, aPKC, and Par-6 form a complex to regulate epithelial polarity. In mammalian cells, this complex localizes above AJ at tight junctions (Nelson 2003). Considering aPKC and Par-6 can bind Baz (Hutterer et al. 2004; Wodarz et al. 2000), it was hypothesized that they might also localize to apical membrane domain in this context. Thus, the Par complex localization in the mid-stage of pupal developing eyes was examined (Hong et al. 2003; Nam and Choi 2003). Surprisingly, Baz localizes at the AJ (Hong et al. 2003; Nam and Choi 2003), but Par-6/aPKC localize at the apical membrane

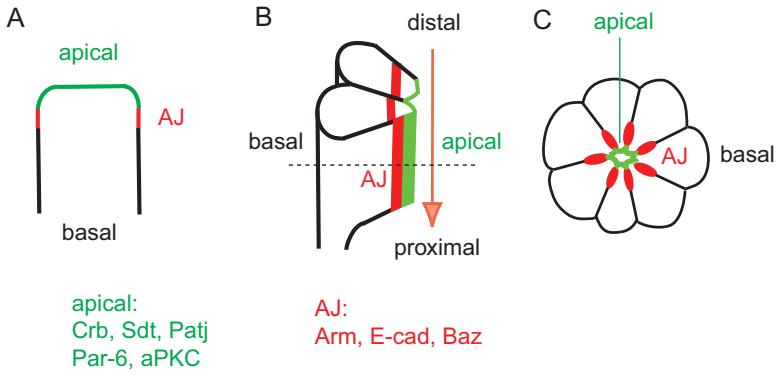

Fig. 2 Localization of Crb and Par complexes in *Drosophila* photoreceptors. (**a**) Schematic view of developing eye disc cell in third-instar larvae. Apical domains of photoreceptors (green) face the retinal surface and are held together by the AJ (red). (**b**) Longitudinal section of a photoreceptor cluster in mid-pupal stage. At this stage, the apical domain (green) and the AJ are oriented towards the center of the ommatidial cluster, as photoreceptors have rotated 90° inward during early pupal stage. (**c**) Tangential section was indicated by the dashed line in (**b**). In tangential section of mid-pupal photoreceptors shows the apical domains (green) face into the center and surrounded by the AJ (red). All of the Crb complex (Crb/Sdt/Patj) and Par-6/aPKC of Par complex localize at the apical domain (green), whereas Baz localizes at AJ (red) with Armadillo (Arm, β-Catenin homolog) and E-cadherin (E-cad)

domain, *Drosophila* homolog of tight junction. Also, the Crb/Sdt/Patj, a Crb complex, localize at the apical domain (Nam and Choi 2003). Therefore, all of the Crb and Par complex localize at the apical domain, except the Baz (Fig. 2).

Previously, it is reported that the apical domain and AJ domain are controlled by the intracellular domain of Crb (Izaddoost et al. 2002; Klebes and Knust 2000). Ectopic expression of Crb^{JM} mislocalized the AJ, but Crb^{PBM} mislocalized the apical domain, respectively (Izaddoost et al. 2002). Using this independent mislocalization of apical and AJ driven by Crb misexpression, the localization of Baz at AJ was further examined (Nam and Choi 2003). Not only the Baz localize at AJ, but also Baz was recruited together with AJ to ectopic membrane sites by misexpression of Crb^{JM}, suggesting that Baz is an integral component of AJ (Nam and Choi 2003). However, Baz is not recruited by Crb^{PBM}, whereas Par-6 and aPKC can be ectopically recruited by Crb^{PBM} rather than Crb^{JM}. Therefore, Baz appears to be recruited to AJ independently of Par-6/aPKC (Nam and Choi 2003).

Intriguingly, despite its specific localization to AJs, loss of Baz resulted in most severe disruption of AJ as well as the more apical domain (Hong et al. 2003; Nam and Choi 2003; Nam et al. 2007). Therefore, the result that *baz* mutation causes loss of Par-6/aPKC, Crb/Sdt/Patj and AJ supports the crucial role of Baz in the initial step of cell polarization (Nam and Choi 2003; Nam et al. 2007). However, the distinct localization of Baz from Par-6/aPKC in the photoreceptors suggested that Baz might be targeted to the membrane with Par-6 but be sorted out from Par-6 in subsequent steps of polarization to remain in the AJs (Choi et al. 2007; Nam and Choi 2003, 2006).

Furthermore, the localization of Baz at AJ is not the pupal stage specific. The Baz localizes at the AJ in the early larval eye discs, and doesn't overlap with the apical domain on which the other polarity components localize (Nam and Choi 2003).

The initial finding of Baz at AJ and its separate localization from Par-6/aPKC in the larval and pupal eyes discs (Hong et al. 2003; Nam and Choi 2003) was confirmed in early epithelia cells in *Drosophila* embryo where epithelial cells first form (Harris and Peifer 2004, 2005). Early in cellularization, Baz colocalizes with AJs rather than aPKC or Par-6. At gastrulating embryos, Baz continues to colocalize with AJ in the epidermis (Harris and Peifer 2004) and in the posterior midgut invagination. aPKC remains apical to AJs in both tissues, whereas PAR-6 becomes enriched above Baz, colocalizing with aPKC in the extreme apical domain. Thus, most cortical Baz remains segregated from aPKC and PAR-6 during gastrulation and retains close AJ association. Baz was previously found to localize above AJs at stage 14 when the epithelium is fully polarized (Wodarz et al. 2000). This was reexamined (Harris and Peifer 2004, 2005) and found some segregation at stages 11 and 12 that became more pronounced at stage 14 and later. At stage 14, segregation was most evident in the gut and in segmental furrows of the epidermis. In each case, Baz appears to localize just apical to AJs. However, PAR-6 continues to localize just apical to Baz. Thus, in late stage epithelia, the apical domain is stratified into three regions, the apical and "mid-apical" and the AJ (Harris and Peifer 2004, 2005). Previous models placed AJs at the top of the epithelial polarity establishment hierarchy (Drubin and Nelson 1996). However, it was found that Baz establishes apical complexes along cellularization furrows in the absence of AJs, and that Baz is required for recruiting AJ into apical spot junctions. These results show that Baz acts upstream of AJs as epithelial polarity is established during *Drosophila* cellularization (Harris and Peifer 2004, 2005).

Localization of Baz Is Controlled by Par-1 Kinase and PP2A Phosphatase

As Par-1 kinase activity is important for inducing Baz mislocalization, and Baz is a biochemical substrate for Par-1 protein kinase (Benton and St Johnston 2003), it is possible that Par-1 phosphorylation of Baz may be responsible for the mislocalization of AJ and apical markers. To test this possibility, GFP-tagged wild-type Baz (Baz^{WT}) was expressed in differentiating retinal cells and examined whether the GFP-Baz proteins are normally localized to AJ or are recruited to ectopic positions in the photoreceptor cells (Choi et al. 2007; Nam et al. 2007). Most GFP-Baz^{WT} was severely displaced to apical or basolateral regions. The apical marker was also diffused and mislocalized basolaterally from the apical domain, implying the disruption of apical-basal cell polarity (Choi et al. 2007; Nam et al. 2007). But, the unphosphorylatable Baz by Par-1 having mutations at the Par-1 phosphorylation sites was predominantly localized to the normal AJ positions basal to the apical domain (Choi et al. 2007; Nam et al. 2007).

Furthermore, the phosphorylation of Baz by Par-1 was dephosphorylated by protein phosphatase 2A (PP2), therefore, the localization of Baz at AJ is controlled by Par-1 phosphorylation and PP2A dephosphorylation (Choi et al. 2007; Nam et al. 2007). The dephosphorylation of Baz by PP2A and its antagonistic function against the Par-1 was further confirmed in *Drosophila* neuroblasts polarity (Krahn et al. 2009).

Separation of Baz from Par-6/aPKC Is Triggered by Crb and aPKC

Baz, PAR-6, and aPKC form a complex that plays a key role in the polarization of many cell types and cell polarity-dependent organ morphogenesis. In epithelial cells including the photoreceptors (Hong et al. 2003; Nam and Choi 2003) and embryo (Harris and Peifer 2004, 2005), however, Baz localizes at AJ below PAR-6 and aPKC. Recently, the molecular separation mechanism of Baz from Par-6/aPKC was discovered (Morais-de-Sa et al. 2010). The mechanism is that Baz is excluded from the apical Par-6/aPKC domain in epithelia by aPKC phosphorylation, which disrupts the Baz/aPKC interaction (Morais-de-Sa et al. 2010). Removal of Baz from the Par-6/aPKC complex also requires the Crb complex, which prevents the Baz/PAR-6 interaction. In the absence of Crb or aPKC phosphorylation of Baz, mislocalized Baz recruits AJ components apically, leading to a loss of the apical domain and an expansion of lateral (Morais-de-Sa et al. 2010) (Fig. 3). This molecular sorting mechanism of Baz from Par-6/aPKC was also confirmed in developing pupal photoreceptor (Walther and Pichaud 2010). Furthermore, the molecular sorting mechanism of Baz was proposed as an apical boundary establishment during the photoreceptor polarity remodeling during the pupal eye development. However, the separation of Baz from Par-6/aPKC and Crb complex is not unique feature in pupal eyes, since the same separations were found in developing earlier larval eyes (Nam and Choi 2003), follicles cells (Morais-de-Sa et al. 2010) and embryos (Harris and Peifer 2004, 2005). There is a possibility of PP2A as a negative regulator against the aPKC on Baz, since the antagonistic relationship between aPKC and PP2A was found in *Drosophila* neuroblast (Chabu and Doe 2009; Ogawa et al. 2009) and mammalian epithelia (Nunbhakdi-Craig et al. 2002) (Fig. 3).

FERM (Band 4.1, Ezrin, Radixin, Moesin) Proteins in Retina

Crb has a highly evolutionary conserved short cytoplasmic tail that contains a PDZ domain binding site (PBM) at its C terminus and a juxtamembrane region (JM) that was predicted to act as a FERM domain binding site (Klebes and Knust 2000) (Fig. 3). Both PBM and JM are important for the function of Crb in retina (Izaddoost

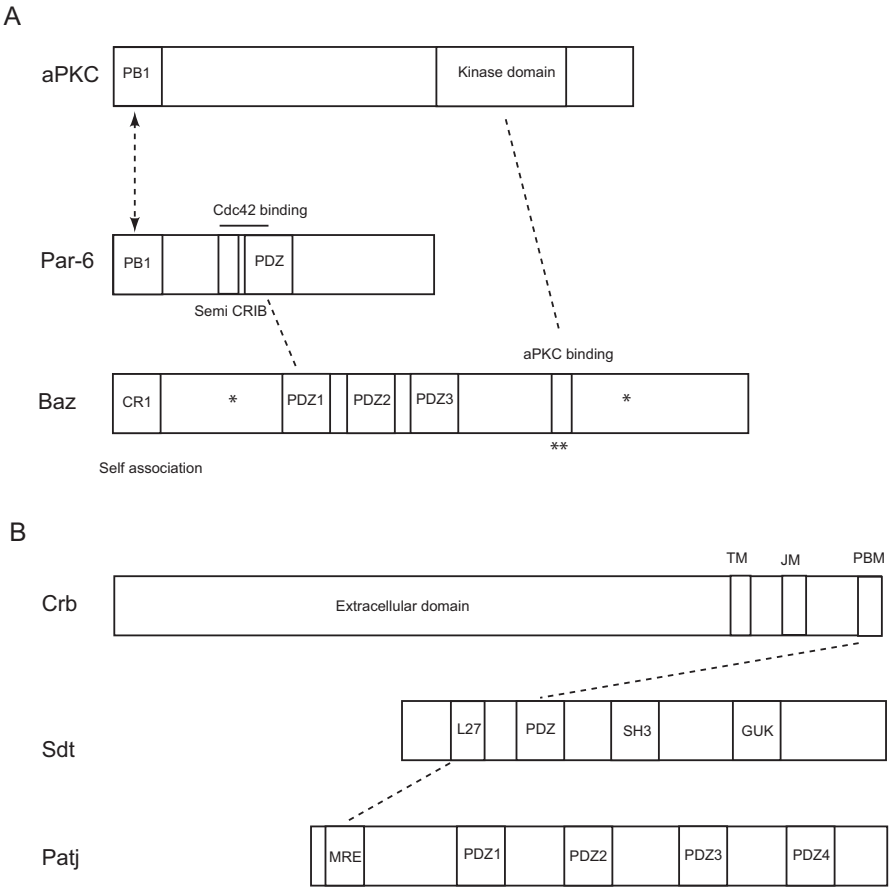

Fig. 3 The Par and Crb complexes and their interactions. (**a**) Domain organization of aPKC, PAR-6, and Baz. The phagocyte oxidase/Bem1 (PB1) domain binds other PB1 domains. PSD-95, Discs large, Zona occludens-1 (PDZ) domain binds other PDZ or PDZ-binding motif (PBM). CDC42/Rac-interactive binding (CRIB) domain binds a small GTPase in GTP-bound state (the CRIB domain of PAR-6 is not sufficient enough, so is referred to as a "semi-CRIB" domain). An aPKC-binding domain in PAR3 is phosphorylated by the kinase. The amino-terminal conserved region (CR1) is required for oligomerization of Baz. Single asterisks (∗) indicate the Par-1 phosphorylation sites, and double asterisk (∗∗) indicates the aPKC phosphorylation site in Baz. (**b**) Domain organization of Crb, Sdt, and Patj. Crb has a transmembrane domain (TM), juxtamembrane (JM), and PBM. Guanylate kinase (GUK) is a protein binding domain. Lin2 and Lin 7 (L27) domain binds MAGUK recruitment element (MRE) domain. Dashed lines indicate regions of the proteins that interact with one another

et al. 2002). However, the PBM recruit the apical stalk membrane; in contrast, the JM control the AJ, respectively (Izaddoost et al. 2002). The JM has a potential FERM binding sequence, therefore, it was postulated that a FERM protein will control the AJ through the JM of Crb (Izaddoost et al. 2002). Two FERM proteins were suggested as potential candidates for mediating this interaction (Karagiosis

and Ready 2004; Laprise et al. 2006). One of the FERM proteins is Moesin. Moesin localizes to rhabdomere base, and is essential for the apical membrane and rhabdomere (Karagiosis and Ready 2004). The other FERM protein is Yurt that localizes at the basolateral membrane domain. It transiently localizes to the apical stalk membrane during a very late stage of pupal eye development. Yurt negatively regulates Crb since *yurt* mutants show an apical membrane expansion similar to Crb overexpression (Laprise et al. 2006). Although the roles of FERM proteins of Mosein and Yurt are clear on the Crb in the retinal development, the evidence of the Moesin/Yurt's role on Crb-AJ linkage was not conclusive. However, recent data strongly support the role of Yurt in Crb's AJ regulation (Salis et al. 2017). Furthermore, recent discovery of a reciprocal antagonistic regulation between aPKC and Yurt in the segregation of distinct and mutually exclusive membrane domain further supports the important role of Yurt in cell polarity regulation (Gamblin et al. 2014). The zebrafish gene *mosaic eyes* is a homolog of Yurt (Jensen and Westerfield 2004). The *mosaic eyes* mutation in zebrafish retina showed a retinal junction phenotype (Christensen and Jensen 2008; Hsu et al. 2006; Jensen and Westerfield 2004).

Role of LKB1 (Par-4), AMPK

LKB1 (Par-4) is essential for the correct distribution of polarity determinants during early embryo development in *Caenorhabditis elegans* (Morton et al. 1992; Watts et al. 2000) and *Drosophila* (Martin and St Johnston 2003). Recently, the role of LKB1 (Par-4) was found to have an essential role in apical junction and AJ regulation in the pupal retina morphogenesis (Amin et al. 2009). Especially, the loss of LKB1 caused the mixing of the apical domain and the basolateral domains, which is a cell polarity defect (Amin et al. 2009).

Surprisingly, AMPK was found to be dispensable in retinal development (Amin et al. 2009; Spasic et al. 2008). Further, it was found that LKB1 does not act primarily through AMPK to regulate cell polarity in the retina (Amin et al. 2009), although it was known that LKB1 acts primarily through the AMP kinase to establish and/or maintain cell polarity in other system or organ (Lee et al. 2007; Mirouse et al. 2007). But, it was found that AMPK was crucial to maintain the adult *Drosophila* photoreceptor from the neurodegeneration caused by light-induced excitation (Spasic et al. 2008). Further, energy depletion in *ampk* mutants resulted in increase of autophagy, and promotion of neurodegeneration in *Drosophila* retina (Poels et al. 2012).

Role of Phosphatidylinositol Lipids in Retina Morphogenesis

The apical and basolateral surfaces of the cell have completely different protein and lipid compositions, and so the cell has mechanisms to specifically sort these components to one surface or the other. The lipids phosphatidylinositol bisphosphate

(PIP2) and phosphatidylinositol triphosphate (PIP3) are crucial determinants of the identities and formation of the apical and basolateral surfaces, respectively (Bryant and Mostov 2008; Martin-Belmonte and Mostov 2007). PIP2 localizes at the apical membrane domain, and PIP3 localizes at the basolateral membrane domain, respectively (Bryant and Mostov 2008). Phosphatidylinositol 3-kinase (PI3K) converts PIP2 to PIP3, and phosphatase and tensin homolog (PTEN) converts PIP3 to PIP2 (Di Paolo and De Camilli 2006; Gassama-Diagne et al. 2006; Martin-Belmonte and Mostov 2007).

In developing mid-pupal photoreceptors, PIP3 was enriched in the whole apical membrane. The PIP3 was later restricted into the rhabdomere in the late pupal photoreceptors (Pinal et al. 2006). In contrast, PIP2 was mainly localized in the AJ, as well as the apical and basolateral domains at lower levels (Pinal et al. 2006). Therefore, the appropriate control of the PIP3 levels in the cell membrane may be required to define the apical rhabdomere area (Pinal et al. 2006).

Trafficking and Secretion During Retina Morphogenesis

During retina development in pupal stage, a massive trafficking from cytoplasm to apical membrane domains is essential. Therefore, cellular trafficking components were postulated to involve the rhabdomere elongation and growth. Rab11, a small GTPase implicated in membrane traffic, in the trans-Golgi network, cytoplasmic vesicles, and the rhabdomere base (Satoh et al. 2005). When Rab11 activity is reduced, rhabdomere morphogenesis was inhibited. Then, it was proposed that Rab11 has a role in the post-Golgi transport to the rhabdomeric membranes of photoreceptors. Furthermore, other exocytosis genes of Sec6, Sec8, and Sec15 were identified to be involved in the rhabdomere morphogenesis (Beronja et al. 2005; Li et al. 2007; Wu et al. 2005). However, cell polarity protein targeting was not affected in the absence of the Sec6 (Beronja et al. 2005). Therefore, the targeting of the cell polarity proteins to the membrane may be independent from the exocytosis (Fig. 4).

Role of Spectrins in Membrane Domain Modulations

Spectrins are major proteins in the cytoskeletal network of most cells. In *Drosophila*, β_{Heavy}-spectrin encoded by *karst* gene functions together with Crb during photoreceptor morphogenesis (Pellikka et al. 2002). Recently, it has been shown that Karst colocalizes with Crb at the rhabdomere stalk, and interacts with Crb (Medina et al. 2002; Pellikka et al. 2002). The rhabdomere stalk length is reduced in photoreceptors of *karst* mutant adult eyes. This phenotype was enhanced by the presence of one copy of *crb* mutation, indicating that *karst* and *crb* genetically cooperate for rhabdomere stalk maintenance. The rhabdomere stalk localization of Karst depends on Crb (Pellikka et al. 2002). While Karst localizes apically, β-spectrin is preferentially

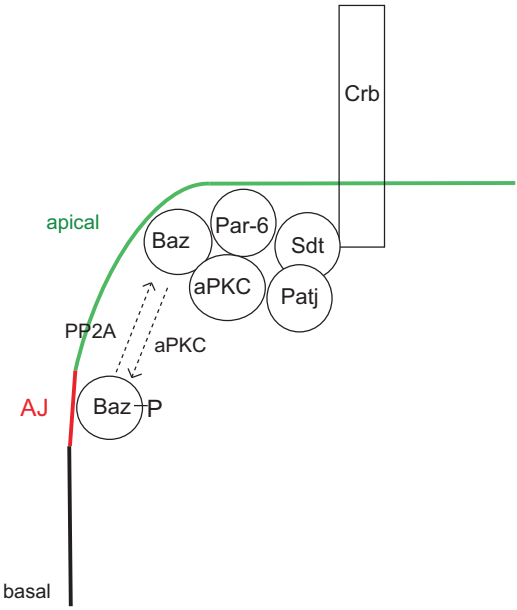

Fig. 4 Baz localization at AJ from Par-6/aPKC and Crb complexes. Par-6 and Crb complex (Crb/Sdt/Patj) proteins are targeted to the apical region of epithelia cells, except that Baz is localized to AJ (red) between the apical (green) and the basolateral domains. Baz protein phosphorylated by aPKC at the S980 aPKC site causes the Baz separation from the apical Baz/Par-6/aPKC complex, thereby the Baz is localized to AJ. The PP2A phosphatase antagonizes aPKC kinase function by dephosphorylating Baz

distributed in the basolateral region (Chen et al. 2009). Overexpression of the basolateral β-spectrin caused a strong shrinkage of apical membrane domains, and loss of the β-spectrin causes an expansion of apical domains, implying an antagonistic relationship between β-spectrin and karst. These results indicate that spectrins are required for controlling photoreceptor morphogenesis through the modulations of apical and basolateral cell membrane domains (Chen et al. 2009).

Function of Actin Cytoskeleton in Retina Morphogenesis

Drosophila photoreceptors undergo massive elongation during pupal development (Fig. 1). This elongation includes the rhabdomere elongation. The rhabdomere is a stabilized actin cytoskeleton, therefore, it was postulated that the actin cytoskeleton might provide an important role in this elongation step. Recently, cofilin/ADF (actin-depolymerizing factor) was found to be required for this process (Pham et al. 2008). Further, Abelson (Abl) tyrosine kinase, a regulator of actin cytoskeleton, was found to be an essential role for this rhabdomere elongation step (Xiong and Rebay 2011). However, there was no direct evidence between the actin cytoskeleton and the cell polarity genes, yet. WASP (Wiskott–Aldrich syndrome protein) and CYFIP/Sra-1 (a member of the WAVE/SCAR complex and regulator of actin remodeling) were suggested and identified as regulators of the actin-based rhabdomeres biogenesis (Galy et al. 2011; Zelhof and Hardy 2004). Furthermore, myosin V was found

to involve the material trafficking for the rhabdomere components (Li et al. 2007), and to be stabilized by the Crb for the rhodopsin trafficking (Pocha et al. 2011).

Studies in one-cell stage embryo of *C. elegans* suggest that PAR polarity complex segregation relies on asymmetric actomyosin activity (Kemphues 2000). However, the mechanism of activation of apically polarized actomyosin contractility was unknown. Recently, it was found that the myotonic dystrophy-related Cdc42-binding kinase (MRCK) activates myosin-II at the apical membrane to segregate aPKC–Par6 from junctional Par-3/Baz, defining the apical membrane domain (Zihni et al. 2017). Thus, MRCK-activated polarized actomyosin contractility is required for apical morphogenesis in Drosophila photoreceptors. This result identified an apical origin of actomyosin-driven morphogenesis that couples cytoskeletal reorganization to PAR polarity signaling (Wang et al. 2017; Zihni et al. 2017).

Stable/Acetylated Microtubules in *Drosophila* Retina

In animal photoreceptor cells, the surface membrane is enlarged for the storage of opsin photopigment. Insect eyes use an actin-based structure for surface membrane enlargement, but mammalian eyes use microtubule-based structure (Land and Nilsson 2002). Previously, the microtubules in developing early *Drosophila* photoreceptors were reported in developing eye discs during the third-instar larval stage (Corrigall et al. 2007; Fernandes et al. 2014; Lei and Warrior 2000; Mosley-Bishop et al. 1999; Whited et al. 2004). Nuclear positioning or migration functions were defects in the larval eye discs in mutants in microtubule-dependent genes including *klarsicht* (Mosley-Bishop et al. 1999), *dynactin* (Whited et al. 2004), *lissencephay1* (Lei and Warrior 2000), *spastin* (Corrigall et al. 2007), and EB1 (Fernandes et al. 2014). However, the functional role of microtubules in rhabdomere morphogenesis was not reported. Previously, a subcellular localization of a microtubule structure at the base of the rhabdomere was identified in the pupal photoreceptors (Fan and Ready 1997). Further, the microtubules at the rhabdomere base were recently identified as stable/acetylated microtubules (Chen et al. 2010). Given the specific localization of stable microtubules in developing pupal photoreceptors (Fig. 5), these subcellular structures might provide a functional role for photoreceptor morphogenesis.

Role of Spastin in *Drosophila* Retina Elongation

Spastin is a microtubule-severing AAA ATPase involved in constructing neuronal and non-centrosomal microtubule arrays (Lumb et al. 2011; Roll-Mecak and McNally 2009; Salinas et al. 2007). In mammals, spastin has been shown to modulate the microtubule cytoskeleton (Errico et al. 2002). The *spastin* mutation in developing pupal eyes causes a mild mislocalization of the apical membrane domain

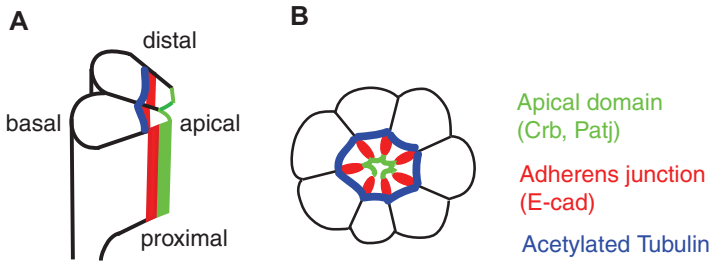

Fig. 5 Schematic diagram of the localization of stabilized microtubules in pupal photoreceptors. The apical markers (Crb) localize at the apical domain (green). The E-cad localizes at AJ (red) which are more basal to the apical domain. The acetylated-tubulin (blue) localizes at the outside from the AJs (red)

at the distal section, but the apical domain was dramatically reduced at the proximal section of the developing pupal eye (Chen et al. 2010). Since the rhabdomeres in developing pupal eyes grow from distal to proximal (Izaddoost et al. 2002), this phenotype strongly suggests that *spastin* is required for apical domain maintenance during rhabdomere elongation. This role of *spastin* in apical domain modulation was further supported by *spastin's* gain-of-function phenotype. Spastin overexpression in photoreceptors caused the expansion of the apical membrane domain from apical to basolateral in the developing photoreceptor (Chen et al. 2010). Although the localizations of the apical domain and AJs were severely expanded, there were no defects in cell polarity. These results strongly suggest that spastin is essential for apical domain biogenesis during rhabdomere elongation in *Drosophila* photoreceptor morphogenesis.

Role of Centrosomin (Cnn) in Drosophila Retina Morphogenesis

Cnn is a core protein for centrosome that is a major microtubule-organizing center. The effect of the *cnn* mutation on developing eyes was recently reported (Chen et al. 2011). Photoreceptors deficient in Cnn displayed dramatic morphogenesis defects including the mislocalization of Crb and Baz during mid-stage pupal eye development, suggesting that Cnn is required for photoreceptor morphogenesis during pupal eye development. This role of Cnn in apical domain modulation was further supported by Cnn's gain-of-function phenotype. Cnn overexpression in photoreceptors caused the expansion of the apical Crb membrane domain, Baz and AJs (Chen et al. 2011). These results strongly suggest that the interaction of Baz and Cnn is essential for apical domain and AJ modulation during photoreceptor morphogenesis.

Role of Kinesin Motors in *Drosophila* Retina Morphogenesis

Crb, a cell polarity gene, has been shown to provide a positional cue for the extension of the apical membrane domain, AJ, and rhabdomere along the growing proximal-distal axis during *Drosophila* photoreceptor morphogenesis (Izaddoost et al. 2002; Pellikka et al. 2002). In developing *Drosophila* photoreceptors, a stabilized microtubule structure was discovered (Chen et al. 2010; Fan and Ready 1997) and its presence was linked to polarity protein localization (Chen et al. 2010). It was therefore proposed that the microtubules may provide trafficking routes for the polarity proteins during photoreceptor morphogenesis (League and Nam 2011). Recently, Crb localization was examined in the developing photoreceptors of *kinesin-1* mutants (League and Nam 2011). The *kinesin-1* mutant photoreceptors showed a range of abnormalities in the apical membrane domain depending on the position along the proximal-distal axis in pupal photoreceptors (League and Nam 2011). The *kinesin-1* mutant showed a progressive mislocalization in the apical domain along the distal-proximal axis during rhabdomere elongation (League and Nam 2011). The *kinesin-1* mutation also led to a similar progressive defect in the stabilized microtubule structures, strongly suggesting that kinesin-1 motor is essential for microtubule structure and Crb localization during distal to proximal rhabdomere elongation in pupal morphogenesis. This role of kinesin-1 in apical domain control was further supported by *kinesin-1*'s dominant-negative mutation phenotypes, which showed disruption of the apical membrane domain and the stabilized microtubules in the developing photoreceptors (League and Nam 2011). These phenotypes suggest that kinesin-1 is essential for the microtubule structures and apical membrane domains during the distal-proximal elongation of photoreceptors, but is dispensable for early eye development.

Another kinesin motor, kinesin-2, was analyzed in photoreceptor development (Mukhopadhyay et al. 2010). It turned out that kinesin-2 is essential for viability of developing photoreceptors and localization of junctional proteins during early eye development of eye disc differentiation (Mukhopadhyay et al. 2010). The early *kinesin-2* mutants cause abnormal nuclear position in differentiating photoreceptors. These cells eventually die in the pupal stage, indicating kinesin-2's role in cell viability. Further, kinesin-2 was essential for Baz localization to the AJ in pupal photoreceptors (Mukhopadhyay et al. 2010). These findings suggest that kinesin-2 motor plays a primary role in the localization of AJ and cell polarity proteins in the developing retina (Mukhopadhyay et al. 2010). In contrast, the kinesin-1 motor participates in a more specific step of apical domain elongation during the rhabdomere morphogenesis (League and Nam 2011). Kinesin-2's function in mouse retina was reported. The absence of kinesin-2 caused optin trafficking defects and followed by cell death (Avasthi et al. 2009; Jimeno et al. 2006a, b; Lopes et al. 2010).

Role of Tau, a Microtubule-Associated Protein, in Drosophila Photoreceptor Morphogenesis

Based on the presence of acetylated and stabilized microtubule cytoskeleton in developing photoreceptors and its role in photoreceptor cell polarity, microtubule-associated proteins might have important roles in controlling cell polarity proteins' localizations in developing photoreceptors. Tau, a microtubule-associated protein, was identified to have a crucial role in photoreceptor cell polarity (Nam 2016). Tau colocalizes with acetylated/stabilized microtubules in developing pupal photoreceptors. Although the Tau is dispensable in early eye differentiation and development (Bolkan and Kretzschmar 2014), it turned out that the Tau has an essential role in late stage of photoreceptor polarity (Nam 2016). The absence of the Tau caused the substantial reduction of the polarity proteins' targeting to the apical membranes. The Tau's role in photoreceptor cell polarity was further supported by Tau's overexpression studies. Tau overexpression caused dramatic expansions of apical membrane domains where the polarity proteins localize in the developing pupal photoreceptors. It was also found that Tau's role in photoreceptor cell polarity depends on Par-1 kinase. It was found that Tau has a crucial role in cell polarity protein localization during pupal photoreceptor morphogenesis stage (Nam 2016), but not in early eye development including eye cell differentiation (Bolkan and Kretzschmar 2014).

Role of Spectraplakin, an Actin-Microtubule Linker, in *Drosophila* Retina Morphogenesis

Coordinated interactions between microtubule and actin cytoskeletons are involved in many polarized cellular processes. Since spectraplakin is able to bind both microtubule and actin cytoskeletons, the role of Short stop (Shot, *Drosophila* homolog of spectraplakin) (Lee et al. 2000; Lee and Kolodziej 2002) was analyzed in the regulations of apical Crb domain in developing *Drosophila* photoreceptors (Mui et al. 2011). The localization pattern of Shot in developing pupal photoreceptors showed a unique intracellular distribution. Shot localized at rhabdomere terminal web (Ready 2002; Xia and Ready 2011) which is at the basal side of the apical Crb or rhabdomere, and in between the AJs (Fig. 6). The rhabdomere terminal web, where the Shot localizes, may be the interface where the stable microtubules and F-actins of rhabdomere meet together. Since Shot has an actin-microtubule crosslinking activity, Shot might cross-link the two cytoskeletons of actin and microtubules at the rhabdomere terminal web. The *shot* mutant photoreceptors showed dramatic mislocalizations of Crb, AJs, and the stable microtubules (Mui et al. 2011). This role of Shot in Crb and AJ regulation was further supported by *shot*'s gain-of-function phenotype (Mui et al. 2011). Shot overexpression in photoreceptors caused a cell polarity defect including dramatic mislocalization of Crb, AJs, and the stable

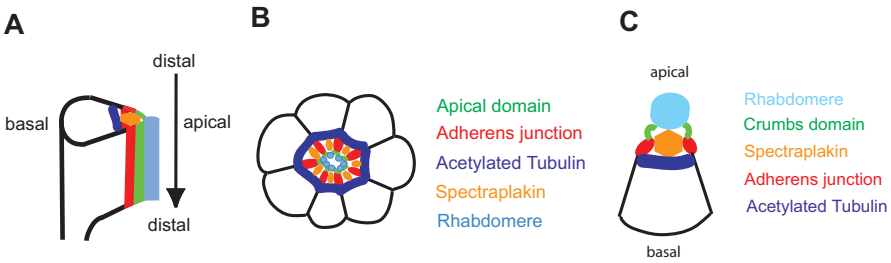

Fig. 6 Schematic representation of mid-pupal photoreceptor and localization of Shot. Shot (orange) localizes in between adherens junction (red), at the basal side of the apical Crumbs domain (green), at the apical side of the stable microtubule (blue), and at the basal side of the rhabdomere (light blue)

microtubules in the developing photoreceptors. This data suggest that Shot, an actin-microtubule cross-linker, is essential in the apical and adherens junction controls during the photoreceptors morphogenesis. A similar role of spectraplakin in mouse retina was also found (May-Simera et al. 2016). Deletion of microtubule actin crosslinking factor 1 (MACF1), a spectraplakin, in developing mouse retina caused defects in ciliogenesis and photoreceptor polarity (May-Simera et al. 2016).

Inter-retina Space Formation During *Drosophila* Retina Morphogenesis

Drosophila has an open rhabdom system, in which the rhabdomeres of each ommatidium are separated from each other (Land and Nilsson 2002). This system evolved from the ancestral insect eye, which has fused rhabdoms. Recently, several genes involving in this rhabdomere separation were recently identified (Husain et al. 2006; Zelhof et al. 2006). Eyes shut (spacemaker), prominin, and chaoptin are responsible to generate inter-rhabdomere space which is an extracellular lumen. Although the eyes shut mutation does not affect the cell polarity defects in retina, the eyes shut is secreted to the inter-retina space through the rhabdomere stalk (Husain et al. 2006) which is controlled by cell polarity genes. Therefore, there is a potential possibility of cell polarity genes' role in eyes shut secretion and then inter-retina space formation.

Surprisingly, several studies identified mutations in a human ortholog of *Drosophila* eyes shut are responsible to cause retinitis pigmentosa (Abd El-Aziz et al. 2008; Collin et al. 2008). These two fundamentally different types of photoreceptors use totally different materials to increase the surface areas for housing photopigments. Vertebrate eyes utilize microtubule-based cilia, but Drosophila eyes use actin-based rhabdomere. However, they use the same way to make inter-retina space in the retina. A similar conserve of prominin was also identified between *Drosophila* and mouse (Nie et al. 2012).

Significance and Perspective

Evolutionary conservation in the structure and function of polarity genes makes the *Drosophila* retina an excellent model for studying the genetic and molecular basis of retinal cell organization and retinal diseases resulting from mutations in polarity genes (den Hollander et al. 1999). For example, mutations in human Crb homolog cause retinal diseases such as a late-onset retina degeneration of retinitis pigmentosa (den Hollander et al. 1999) and an early-onset retinal degeneration of Leber congenital amaurosis (den Hollander et al. 2001). Searching for new genes interacting Crb will help in the search for new therapy targets for these eye diseases. This study may broaden our knowledge of the *Drosophila* photoreceptor, one of the best genetic model systems, and expand its usefulness as a model system for human retina diseases. Based on the strict conservation of genes and cellular structures between *Drosophila* and human retinas, narrowly defined classic "homology"-based approaches might not be enough to understand deeper similarities between two. The new concept of "Deep homology" deals homology in contexts in which structures are not homologous in the classical sense (Shubin et al. 2009). The new concept of "deep homology" could be an appropriate way to fully understand the deeply conserved mechanisms between these two eyes.

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Negative Regulation for Neural Patterning in the *Drosophila* Eye

Kwang-Wook Choi

Introduction

An adult compound eye consists of about 800 unit eyes called ommatidia. The ommatidia are organized in a highly ordered structure and provide an ideal sensory system for genetic dissection of neural development and cellular pattern formation. Each ommatidium contains 8 photoreceptor neurons and a dozen accessory cells including cone cells, pigment cells, and bristles that are formed in a stereotypic pattern (Ready et al. 1976).

The adult eye develops from eye imaginal disc, an epithelial primordium for eye proper and the surrounding head tissues. In the early phase of development, the eye disc grows by cell proliferation without retinal differentiation. This growth phase continues until retinal differentiation is initiated at the early third instar larval stage. Among several different cell types in the adult eye, photoreceptor neurons are the first kind to be generated in the eye disc. The initial pattern of these cells provides the structural foundation for the subsequent patterning events to establish the adult eye. Thus, how these initial events are organized at the cell and molecular levels is an important question in eye development.

Following the growth phase of eye disc development, retinal neurogenesis is initiated in the morphogenetic furrow (the furrow in short), a groove formed along the dorsoventral axis of the eye disc. This furrow is first generated at the posterior margin of the eye disc and progresses anteriorly during neurogenesis. Importantly, columns of neuronal cell clusters are generated immediately posterior to the furrow (Ready et al. 1976). Thus, the furrow is an important site for retinal organization

K.-W. Choi (✉)
Department of Biological Sciences, Korea Advanced Institute of Science and Technology, Daejeon, South Korea
e-mail: kchoi100@kaist.ac.kr

A. Singh, M. Kango-Singh (eds.), *Molecular Genetics of Axial Patterning, Growth and Disease in Drosophila Eye*,
https://doi.org/10.1007/978-3-030-42246-2_6

where multiple cell signaling pathways are coordinated to specify the founder cells for photoreceptor neurons.

A critical event for retinal neurogenesis in the furrow is to induce the expression of proneural genes like *atonal* (*ato*) that promotes neural differentiation. Ato expression in the furrow is dynamically induced by positive genetic factors expressed within the furrow or adjacent regions anterior or posterior to it. Secreted signaling molecules like Decapentaplegic (Dpp) and Hedgehog (Hh) act as positive factors to induce Ato expression (Baker and Yu 1997; Borod and Heberlein 1998; Greenwood and Struhl 1999). While these factors act as positive regulators of retinal differentiation, spatial patterning of neurogenesis is also dependent on the function of specific negative regulators. The ommatidial pattern consists of repetitive arrays of photoreceptor clusters. Each of these clusters is surrounded by non-neuronal interommatidial cells, resulting in the formation of a precise polka dot pattern in the eye (Fig. 1a). Thus, the function of negative factors in neurogenesis is not only essential for the establishment of the ommatidial pattern but also provides necessary conditions for subsequent differentiation of non-neuronal interommatidial cells in the eye.

One of the key negative regulators of Ato induction is Notch. Notch is a transmembrane protein that functions as a receptor for the membrane-bound ligands, Delta (Dl) and Serrate (Ser). Notch signaling is required at multiple steps during eye development (Cagan and Ready 1989). Notch initially promotes neural differentiation but later antagonizes it by lateral inhibition in the cells surrounding the Ato-expressing cells (Baker and Yu 1997; Baonza and Freeman 2001). In addition, other

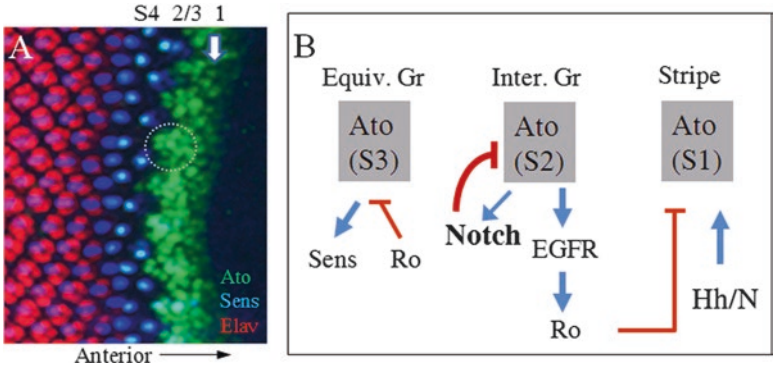

Fig. 1 Regulation of Ato expression. (**a**) Ato expression pattern in eye disc. Ato is expressed initially in a stripe pattern at and immediately anterior to the furrow (stage 1). The stripe pattern is resolved into the intermediate groups (stage 2) and the equivalence groups (stages 3). Later, Ato is expressed only in R8 cells (stage 4). Ato expression in the stages 1 and 2–4 are controlled by the 3′ and 5′ regulatory region, respectively. (**b**) A simplified diagram for gene functions involved in early stages of neurogenesis. The initial Ato expression (S1) is regulated by positive factors including Hh, Dpp, and N, which leads to autoactivated *ato* expression in the stage S2. The S2 Ato induces EGFR/MAPK signaling which inhibits the S1 stage *ato* expression, resulting in evenly spaced intermediate groups. One cell from each R8 equivalence group maintains Ato expression to become a R8 while Ato expression in other cells is repressed by Ro

negative regulators such as EGF receptor (EGFR) and Bar transcription factors have been identified as inhibitory factors for retinal neurogenesis within or behind the furrow. In this chapter, the role of a few key positive factors involved in the retinal fate induction will be briefly introduced. Next, the function of negative regulators will be discussed in more detail to illustrate how the interaction of these positive and negative factors leads to the generation of the initial ommatidial pattern in the eye disc.

Retinal Neurogenesis: Positive Regulation of Atonal Expression

Retinal differentiation begins with the formation of the morphogenetic furrow from the posterior margin of an eye imaginal disc. As the furrow progresses anteriorly, columns of R8 founder neurons are generated from the posterior part of the furrow (Wolff and Ready 1991). Subsequently, additional photoreceptor cells are recruited to R8 by specific cell–cell interactions to generate the remaining 7 photoreceptor cells in the order of R2/R5, R3/R4, and R1/6/7. This process of sequential induction of photoreceptor cell fates illustrates the critical role of the initial R8 selection in retinal neurogenesis.

Neurogenesis is promoted by a group of factors called proneural genes that encodes bHLH family transcription factors. Generation of the R8 founder cells from undifferentiated cells requires the function of proneural gene *ato* at the furrow (Jarman et al. 1994). Ato protein is a homolog of mammalian Ato7 (also called MATH5) (Brown et al. 1998). The expression of Ato protein is transient and dynamically regulated at the furrow, suggesting that *ato* is subject to negative regulation. Ato expression can be divided into 4 stages based on the distinct pattern of expression (Fig. 1): (1) first expression in a stripe pattern across the disc in the most anterior region of the furrow, (2) expression in about 10 cell clusters called intermediate (or proneural) groups just posterior to the stripe, (3) expression in 2–3 cells of an R8 equivalence group, and (4) selected expression in a single R8 founder cell from each equivalence group (Frankfort and Mardon 2002). Transient expression and sequential restriction of Ato expression in the furrow indicate that *ato* expression is regulated by specific spatial and temporal regulatory factors. Analysis of *ato* regulation has identified two *cis*-regulatory regions responsible for *ato* expression at the furrow (Sun et al. 1998). The eye-specific *ato 3′ cis*-regulatory region controls the early stripe pattern (stage 1) and contains binding sites for transcription factors for retinal determination (Tanaka-Matakatsu and Du 2008; Zhang et al. 2006) while the *5′* regulatory region is responsible for the rest of *ato* expression posterior to the stripe (stages 2–4).

Accumulated evidence indicates that the diffusible factor Hedgehog (Hh) and transmembrane protein Notch (N) provide positive signaling to activate *ato* transcription (Baker and Yu 1997; Dominguez and Hafen 1997; Frankfort and

Mardon 2002; Fu and Baker 2003; Hsiung and Moses 2002). Hh is expressed in all photoreceptors and secreted to act on more anterior cells in the furrow. The stage 2 Ato expression (Fig. 1) induces the expression of Rhomboid family proteins that activate the TGFα family ligand Spitz for EGFR signaling in the adjacent cells.

While Hh and Dpp are secreted factors that promote *ato* expression, eventually *ato* gene must be activated by specific transcription factors. Eyeless (Ey) is a *Drosophila* homolog of Pax-6 that is considered to be a master regulator of eye development (Halder et al. 1995). It is a transcription factor with a homeodomain and a paired domain, and is not only required for eye development but also sufficient to induce ectopic eyes when ectopically expressed in non-retinal tissues. Ey is expressed very early in the embryonic primordium for eye disc and is later detected in the anterior region to the furrow as retinal differentiation begins (Baker and Firth 2011; Kumar and Moses 2001). Since Ey is necessary and sufficient for retinal induction, it may directly activate *ato* transcription in an eye-specific manner. However, *ato* acts as a common proneural gene in the initial stage of development of multiple segment-specific sensory organs, not only the eye but also the auditory organs and stretch receptors. Hence, it has been proposed that these sensory organs may have the same origins (Niwa et al. 2004). In this view, Ato is a common transcription factor necessary for all of these three sensory organs rather than the retina-specific proneural factor. The 3′ region of *ato* is required for Ato expression not only in the eye but also in the Johnston's auditory organ in the antenna and the chordotonal organs (Sun et al. 1998). In fact, protosensory organs are formed by Dpp-dependent Ato expression. Further, two Mad-binding sites were identified that are essential for Ato expression in all three sensory organs. This supported that the target sites for the Dpp signal are conserved during the diversification of sensory organs. Interestingly, ectopic eye formation induced by Ey depends on the presence of Dpp and other signaling molecules, and Ato can be expressed in *ey* mutant if cell death is prevented. These findings led to a model that Ey functions as a downstream or parallel component of Ato rather than as an upstream master control factor for Ato expression and retinal differentiation (Niwa et al. 2004; Treisman 2004).

In contrast to this model, further analysis of *ato 3′ cis*-regulatory sequences has provided evidence that Ato expression in the eye disc is directly regulated by Ey and other so called "retinal determination (RD) genes" like Sine oculis (So) and Dacshund (Dac) (Zhang et al. 2006). Hence, the main role of Dpp in Ato induction in the furrow is to regulate the RD gene expression level (Tanaka-Matakatsu and Du 2008). It is interesting to note that the 3′ *cis*-regulatory region of *ato* consists of multiple modules. Hence, the activation of *ato* expression for sensory organs in different imaginal discs appears to be determined by the modular organization of *ato* regulatory region instead of a common regulatory region for Dpp signaling.

Role of Notch for Lateral Inhibition

The initial stripe pattern of Ato expression is sequentially restricted to proneural groups that are separated by ato-negative cells in the interommatidial space. Notch is a conserved key factor involved in lateral inhibition in diverse developmental processes, especially in neurogenesis (Beatus and Lendahl 1998; Cabrera 1990). In the absence of N function, the lack of lateral inhibition results in the formation of excess number of R8 cells, resulting in more compact spacing of ommatidia (Roignant and Treisman 2009).

Scabrous (Sca) is one of the first genes that were found to be required for proper spacing of R8 founder neurons. Sca is a secreted factor that is released from the R8 cells. Sca is a protein related to fibrinogen that acts as a lateral inhibitor of the R8 differentiation (Baker et al. 1990; Ellis et al. 1994). Sca is expressed early in the furrow and is known to be required for lateral inhibition by N-Dl interaction at the level of intermediate clusters. In the absence of Sca, excess cells are selected to become R8 cells, thus disrupting the normal pattern of R8 spacing. Evidence suggests that Sca functions together with Gp150, a target of protein phosphatase DPTP10D. Loss of Gp150 shows more R8 cells as in *sca* mutant eye. Moreover, single and double mutants for *sca* and *gp150* show similar phenotypes, and both proteins are localized together in the endosomes (Fetchko et al. 2002; Li et al. 2003), suggesting that they work in the same pathway for N signaling. Gp150 acts downstream to Sca in the cells that respond to secreted Sca protein. It has been proposed that N activity is downregulated in the neuronal cells by an endosomal pathway, and Sca and Gp150 are indirectly involved in the activation of N signaling in non-neuronal cells by blocking the endosomal pathway. Gp150 is required for all Sca function identified so far, indicating that Sca-Gp150 pathway is a conserved step for N regulation in diverse developmental events (Li et al. 2003). It remains to be studied, however, whether Gp150 function in this process is regulated by the protein phosphatase activity of DPTP10D.

Additional evidence supports the role of endosomal trafficking for N signaling. Clathrin adaptor protein complex-1 (AP-1) plays a role in sorting of membrane proteins in the Golgi network and endosomes (Kametaka et al. 2012). Loss of AP-1 or its accessory protein like *Drosophila* Aftiphilin results in an intracellular accumulation of Sca and downregulation of N since N degradation is promoted in the lysosomes. Sca protein secreted from R8 cells directly interacts with the N extracellular domain to stabilize it (Powell et al. 2001). Knockdown of AP-1 or Aftiphilin by RNAi causes abnormal pattern of R8 initiation, consistent with the role of AP-1 function of N regulation during R8 specification. Notch signaling is also regulated by endocytosis of the Notch ligand Dl. This process is mediated by ubiquitination of Dl by Neuralized, a RING finger domain protein that functions as an ubiquitin ligase (Lai et al. 2001; Pavlopoulos et al. 2001; Weinmaster and Fischer 2011; Yeh et al. 2001). Monoubiquitinated Dl is internalized for endocytosis in the signal-sending cells. For an unknown mechanism, this endocytosis of Dl is necessary for Notch signaling.

N is a type I single-pass transmembrane protein. When activated by its ligands, it is cleaved by the γ-secretase complex, resulting in the formation of the Notch intracellular domain (NICD) that enters the cell nucleus to promote transcription of downstream target genes (Kopan and Goate 2002). γ-secretase is a multi-subunit complex containing Presenilin (Psn) as the catalytic subunit. Since the catalytic activity of Psn depends on the maturation of a holoprotein by proteolytic cleavage (Annaert and De Strooper 1999), the regulation of Psn maturation is important for N signaling. Loss of Psn causes the lack of lateral inhibition in the furrow, indicating the importance of N processing by Psn for proper ommatidial spacing (Ye et al. 1999).

Cis-inhibition and Ligand-Independent Notch Signaling

In addition to its function in R8 selection, Notch is also involved in the following recruitment of other photoreceptor cells. Posterior to the furrow, emerging photoreceptor clusters in the eye disc are assembled in an asymmetric trapezoidal pattern. These clusters have two opposite forms of chirality in the dorsal and ventral halves of an eye, thus showing a mirror symmetric planar cell polarity (PCP) about the dorsoventral midline (Choi et al. 1996; Ready et al. 1976; Singh et al. 2012). Generation of these chiral forms depends on the specification of two photoreceptors, R3 and R4, from R3/4 equivalent precursor cells. One of these two cells is located closer to the equator and has higher Fz signaling than the polar cell. Interestingly, different Fz activity levels in these two cells leads to the asymmetric activation of N signaling, leading to the generation of R3 and R4 cells with low and high N activation, respectively (Cooper and Bray 1999; Fanto and Mlodzik 1999).

The R8 selection and the subsequent R3/4 specification are distinct processes. However, the asymmetric activation of N in R3 and R4 cells has a similarity to the situation of lateral inhibition in the furrow between the R8 founder cell and the surrounding cells. It is worth noting that Hibris (Hbs), initially found as a protein interacting with Roughest (Rst), a cell adhesion factor, is involved in N signaling not only for R3/4 planar polarity but also for proneural patterning in the furrow. A recent study has identified Hbs as a new factor that functionally and physically interacts with Psn and Nicastrin (Ncs), another component of the γ-secretase complex (Singh and Mlodzik 2012).

Studies on the R3/4 specification led to the finding of new mechanisms for N signaling. In the conventional mechanism, N activation is mediated by specific ligands such as Dl and Ser. Notch activation leads to transcriptional repression of Dl and Neur in the signal receiving cells, whereas Dl and Neur are upregulated in the signal-sending cells (Cooper and Bray 1999; del Alamo and Mlodzik 2006; Fanto and Mlodzik 1999; Tomlinson and Struhl 1999). Alternatively, Fz-Dishevelled (Dsh) complex may inhibit N activation in R3 cells. A recent study has found that Ral, a small Ras-like GTPase, is upregulated in response to Fz activation in the equatorial cells, and the upregulated Ral activity represses Notch activation in a

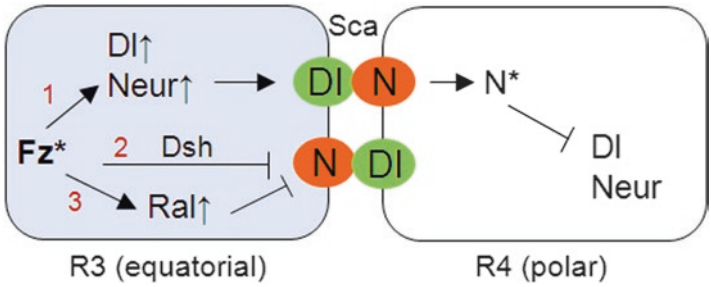

Fig. 2 Regulation of asymmetric Notch signaling. R3/4 precursor cells are initially equivalent. The cell located closer to the equator has more Fz signaling that increases the level of Dl and Neur expression. This cell having more Dl becomes R3. In contrast, the other cell on the polar side becomes R4 with more N signaling. This asymmetric signaling takes place by increased expression of Dl and Neur by Fz (step 1), inactivation of Notch by Fz/Dsh complex (step 2), and/or Fz-dependent upregulation of Ral that inhibits N signaling (step 3). Adapted from Cho and Fischer (2011)

ligand-independent manner (Fig. 2). In this mechanism, Ral GTPase activity may interfere with the ligand-independent Notch activation by regulating Notch trafficking to the lysosome, generation of NICD, or nuclear translocation (Cho and Fischer 2011, 2012).

N signaling is asymmetrically transmitted with directionality because Dl ligand is downregulated in the signal receiving cells by a negative feedback loop (Heitzler and Simpson 1991; Rooke and Xu 1998). In addition to this traditional feedback system, *cis*-inhibition has been proposed as an alternative mechanism for directional activation of N signaling. In photoreceptor recruitment, R1, 6, and 7 cells are the last cells to be specified. R1 and 6 cells are recruited together and express Dl to activate the R7 fate in the neighbor. In this process, Dl ligand expressed in R1 and R6 cells *cis*-inhibits N in the same cell, which prevents inappropriate N activation in R1/6 by Dl ligand from the R7 cell. Such *cis*-inhibition of N signaling by Dl in the signal-sending cell may also function in R3/4 recruitment. It is an intriguing question whether similar *cis*-inhibition and ligand-independent regulation of Notch signaling plays a role in asymmetric N signaling for neural induction at the furrow.

EGF Signaling in Ommatidial Spacing

EGFR signaling is another important mechanism that functions throughout different stages of eye development. EGFR is activated by the positive ligand Spitz (Spi), a TGFα homolog, while it is inactivated by the antagonist, Argos (Freeman 1994; Rutledge et al. 1992; Schweitzer et al. 1995). Similar to the vertebrate EGF receptor, *Drosophila* EGFR is also dimerized upon binding of the Spi ligand and activated by autophosphorylation of the dimer. Activated EGFR triggers the conserved intracel-

lular signaling pathway that involves Ras and MAP kinase (Kumar et al. 1998). Ato expression in R8 cells induces Spi ligand expression that activates EGFR in the neighboring cells (Dominguez et al. 1998). Thus, one of the major functions of EGFR signaling is to activate photoreceptor precursor cells to initiate the retinal differentiation in all photoreceptors except the R8 neuron, the source of EGFR ligand (Dominguez et al. 1998; Yang and Baker 2001).

The role of EGFR in ommatidial spacing during neurogenesis was first implicated by abnormal spacing of photoreceptor clusters caused by *Ellipse* dominant mutations. Ellipse mutations turned out to be alleles of *EGFR*, and it was suggested that EGFR may be important for controlling the ommatidial spacing (Baker and Rubin 1989). The role of EGFR for ommatidial spacing was supported by an analysis of EGFR-dependent Rough (Ro) expression. Ro is a homeobox transcription factor induced by EGFR signaling (Kimmel et al. 1990) and negatively regulates the initial *ato* transcription (Fig. 1b), thus generating the spacing between intermediate groups (Dokucu et al. 1996; Pepple et al. 2008). Analysis of *EGFR* mutant clones supports that EGFR is required for ommatidial spacing (Dominguez et al. 1998). Rhomboid-1 expression induced by Ato in R8 cells is essential for the EGFR activation in nascent ommatidia, which secrete a negative inhibitor like Sca to inactivate Ato expression in the neighboring cells, therefore generating interommatidial spacing. Although EGFR is essential for cell survival, its role for photoreceptor recruitment is largely independent of its function in promoting cell survival. Based on these findings, it has been proposed that the primary function of EGFR is to establish the spatial pattern of ommatidia by regulating R8 spacing (Baonza et al. 2001; Yang and Baker 2001).

However, the role of EGFR in R8 spacing has not been clearly demonstrated probably due to the multiple functions of the EGFR signaling in eye development and different experimental methods used for functional analysis. For instance, an analysis of EGFR function using a temperature-sensitive allele did not support its role for ommatidial spacing. In this approach, a temperature-sensitive allele of *EGFR* called $EGFR^{tsla}$ was used to minimize the defects in cell proliferation. $EGFR^{tsla}$ encodes a mutant protein that becomes quickly inactive or functionally null at the restrictive temperature. Analysis of $EGFR^{tsla}$ mutant clones at the restrictive temperature showed normal rate of furrow progression and normal spacing of R8 cells (Rodrigues et al. 2005), which seems to be contradicting with the previous clonal analysis (Baonza et al. 2001; Yang and Baker 2001).

In the earlier clonal analysis with the *EGFR* null allele, *EGFR* loss-of-function (LOF) clones were generated using a *Minute* (*M*) mutation to generate larger mutant clones. Larger $M^{+/+}$ mutant clones can be generated because $M^{+/+}$ cells have growth advantage compared with the neighboring $M^{+/-}$ cells. Importantly, it was found that the $M^{+/+}$ twin spot has strong non-cell autonomous effects on the $EGFR^{-}$ mutant cells. Thus, it was concluded that the defects in Ato expression and ommatidial spacing in the mosaic clone experiments may be due to effects of the *Minute* rather than the effects of *EGFR* mutation. However, it is still possible that $EGFR^{tsla}$ mutant clones at the restrictive temperature may have a very low but sufficient level of functional EGFR protein for normal R8 spacing. Interestingly, two EGFR ligands,

Spitz and Keren, are redundant for EGFR signaling, but loss of both ligands causes abnormal R8 spacing (Brown et al. 2007). Although this supports the requirements of EGFR signaling for normal R8 spacing, additional studies are needed to draw a definitive conclusion about the role of EGFR signaling for the lateral inhibition of Ato expression and R8 spacing.

Anti-proneural Function of Bar

Ato is transiently expressed in the selected R8 cells and turned off soon in several hours. The inhibition of Ato expression behind the furrow is important to prevent the formation of ectopic photoreceptors while maintaining the ommatidial spacing. This repression is mediated by the *Bar* genes that are expressed posterior to the furrow (Lim and Choi 2003). The first Bar mutation (Bar^{1}) was found as a dominant allele that reduces the eye size (Steinberg and Abramowitz 1938). Bar^{1} is a duplication of the *Bar* gene, suggesting that abnormal overexpression of Bar results in reduction of the eye. Further analysis has shown that *dpp* expression in the furrow is strongly reduced in Bar^{1}. Since Dpp is required for furrow progression, it was suggested that Bar^{1} mutation causes premature furrow stop (Chanut and Heberlein 1997; Curtiss and Mlodzik 2000; Heberlein et al. 1993).

Bar encodes two related and functionally redundant homeodomain proteins, *BarH1* and *BarH2* (hereafter abbreviated as "Bar") that is present in a tandem repeat (Akimaru and Saigo 1991; Higashijima et al. 1992). Expression of Bar is regulated dynamically during eye development. In the eye disc, it is specifically expressed in the nuclei of R1 and R6 photoreceptors and later in primary pigment cells. Consistent with this expression pattern, Bar is important for differentiation of R1, R6, and primary pigment cells (Higashijima et al. 1992).

In addition to these cells, Bar is also expressed in all undifferentiated retinal precursors posterior to the furrow (Higashijima et al. 1992) which can be distinguished by the position of their nuclei. Because the nuclei of differentiating photoreceptors migrate apically while those of undifferentiated cells stay in the basal region (Tomlinson and Ready 1987), these undifferentiated cells posterior to the furrow are referred as the "basal cells" (Fig. 3). Bar expression in these basal cells was shown to be crucial for regulating the neural patterning in earlier steps of eye development (Lim and Choi 2003). *Bar* LOF mutant clones showed ectopic *ato* induction at the transcription level, indicating that Bar acts as a transcriptional repressor of *ato*. It is important to note that loss of Bar has little effect on the spacing of intermediate groups and the selection of R8 from the equivalence group in the endogenous furrow. Hence, Bar is required for repressing the early stripe pattern of *ato* expression, but it may have little effects on N-dependent lateral inhibition of *ato* (Lim and Choi 2003).

Because Bar is important for maintenance of the undifferentiated state of the basal cells, spatial and temporal regulation of Bar expression is crucial for proper eye development. Consistent with the Bar function that represses the Ato expres-

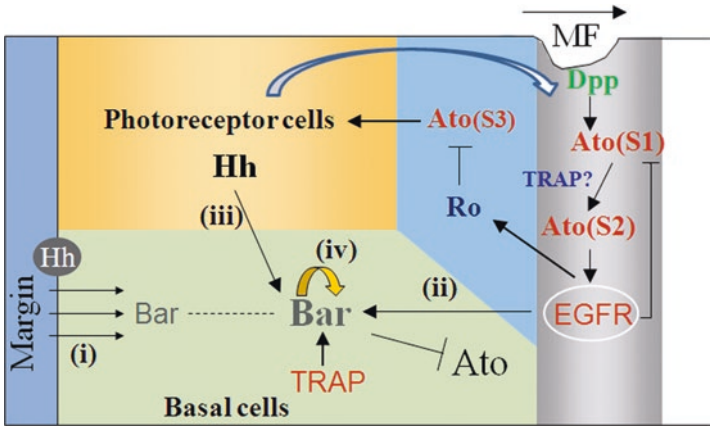

Fig. 3 Bar regulation during retinal neurogenesis. Ato expression in the morphogenetic furrow (MF) is activated by Hh produced by photoreceptor cells and initiates the generation of photoreceptor neurons. Bar proteins are expressed in basal undifferentiated cells behind the furrow (green region) by several mechanisms. Positive and inhibitory relationships indicated by arrows may be indirect: (i) At the time of furrow initiation, Bar expression in the basal undifferentiated cells is induced by a secreted signaling factor, Hh, from the posterior margin (yellow region), (ii) during furrow migration, Bar expression near the furrow is induced by Ato from the furrow. EGFR signaling may partially mediate non-autonomous effects of Ato on Bar expression, (iii) Hh produced in photoreceptor cells induces Dpp expression and may also contribute to Bar expression during furrow migration, and finally (iv) Bar is autoregulated to maintain its expression. Adapted from Lim and Choi (2004)

sion, Bar and Ato expression shows a complementary pattern with a sharp boundary between the Bar^+ and Ato^+ cells along the posterior edge of the furrow. This pattern of Bar expression is regulated by multiple pathways depending on time and position in the disc (Fig. 3) (Lim and Choi 2004). Prior to furrow initiation and Ato expression, Bar appears to be induced by secreted factors from the posterior margin of the disc. For example, Hh is one of the first secreted factor expressed in the posterior margin at the time of furrow initiation, and it has been shown that Hh signaling is in part responsible for initial Bar induction (Lim and Choi 2004). Evidence also suggests that Bar is induced by several factors functioning in the furrow. Immediately behind the furrow, Bar expression depends on EGFR signaling which is induced by Ato expression in the intermediate groups (Fig. 3).

An important function of Ato is to regulate its own expression. Ato protein induced by activation of the 3′ regulatory region can turn on itself by binding to its own 5′ regulatory region (Sun et al. 1998). Interestingly, TRAP (thyroid hormone receptor associated proteins)/mediator complex is involved in the regulation of *ato* expression in the proneural groups. The TRAP complex acts as a coactivator for a variety of transcriptional activators (Ito and Roeder 2001; Malik and Roeder 2000). Among many mediator complex proteins, two *Drosophila* TRAPs, Kohtalo (Kto, TRAP230) and Blind spot (Bli, also called Skuld, TRAP240), have been exten-

sively studied for their roles in retinal neurogenesis. In *TRAP* mutant clones, Ato is ectopically induced behind the furrow (Treisman 2001).

In contrast, TRAPs are required for *ato* expression in the intermediate groups. Because Ato expression in the intermediate groups is dependent on Ato itself, it is possible that TRAP complex might act as coactivator for Ato. Indeed, in the absence of TRAP, Ato fails to induce EGFR signaling and Sca expression that are necessary for lateral inhibition, thus resulting in ectopic Ato expression. Likewise, Kto and Skd are also required for positive Ato functions to induce Ato targets such as Ato itself and Senseless (Sens) within the proneural clusters. Hence, TRAP complex is required for Ato expression and other Ato target genes such as *sca*, *sens,* and *rho* in the intermediate groups (Fig. 4) (Lim et al. 2007).

These studies suggest that proneural and anti-proneural genes function in a negative feedback network. EGFR signaling activated by Ato is necessary for non-autonomous Bar expression near the furrow. In turn, Bar represses *ato* in the basal cells behind the furrow (see a model in Fig. 3). In developing wing disc, TRAP230/240 proteins are necessary for the activation of Wnt signaling and Notch target genes (Carrera et al. 2008; Janody and Treisman 2011). Wg is expressed in the dorsal and ventral margin of an eye disc where it antagonizes the Dpp function for furrow progression. It would be interesting to see whether the TRAP mediator complex also plays a role for Wg regulation and N signaling in the eye.

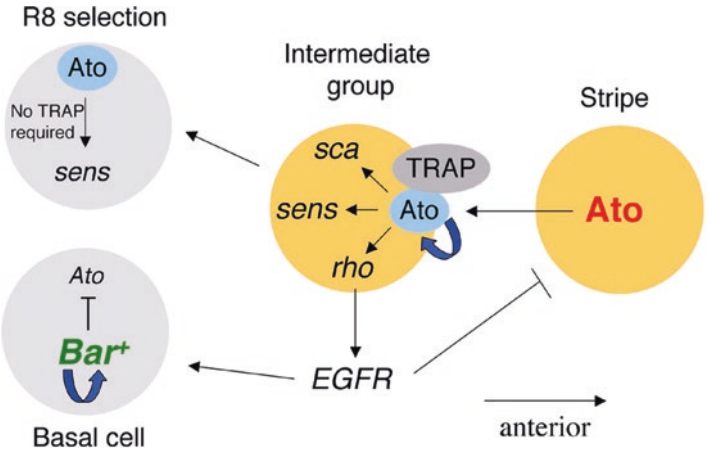

Fig. 4 A model of TRAP-mediated Ato activation in early retinal neurogenesis. Kto (TRAP230) and Skd (TRAP240) function as coactivators for Ato in the proneural clusters and are therefore required for expression of Ato target genes such as Ato itself, Sens, Sca, and for activation of EGFR signaling. Ato and Sens are required for selection and differentiation of the R8 founder neurons. In contrast, EGFR signaling and Sca are involved in repressing Ato expression in cells between proneural clusters. Kto/Skd may not be required for Ato activation in the R8 cells in which Ato activation may depend on other coactivators. In the basal cells, Bar induced by EGFR signaling represses Ato expression. A blue arrow indicates autoregulation. Adapted from Lim et al. (2007)

As described above, Bar and Ato are expressed in a complementary pattern and are antagonistic to each other. It is worth noting that Bar expression is also complementary to Dpp that is expressed in the furrow. Dpp is critical for the initial steps of eye morphogenesis such as furrow initiation, progression, and *ato* activation (Borod and Heberlein 1998; Greenwood and Struhl 1999; Heberlein and Moses 1995). Because *dpp* transcription is induced in the furrow, it provides a marker for the boundary between undifferentiated cells in the anterior domain and differentiating cells in the posterior domain of eye disc. Similarly, *dpp*-expressing cells in limb discs mark the boundary between the anterior-posterior (A/P) compartments (Raftery et al. 1991).

It has been suggested that localized expression of *dpp* at the A/P boundary is largely under negative regulation because sub-fragments of *dpp* regulatory region fused to *lacZ* reporter constructs typically result in ectopic *lacZ* expression rather than its loss (Sanicola et al. 1995). Consistent with this idea, Engrailed (En) homeodomain protein, which acts as the selector for posterior compartments in limb discs (Blair 1992; Kornberg 1981; Morata and Lawrence 1975), is a direct repressor of *dpp*, thus defining the posterior boundary of the *dpp* stripe. In eye development, Dpp is an upstream factor for *ato* induction in the furrow (Fu and Baker 2003; Greenwood and Struhl 1999). Ato expression results in the activation of Bar expression in the region posterior to the furrow indirectly by a combination of EGFR and Hh signaling (Lim and Choi 2004). Since ectopically expressed Bar can repress *dpp-lacZ* in eye and other discs (Lim and Choi 2003), it is possible that Bar expression in the basal cells may be important for preventing ectopic Dpp expression behind the furrow. This negative regulation between *dpp* and *Bar* may play a role in defining the anterior-posterior boundary in developing eye.

Dual Function of Daughterless in Ato Regulation

In addition to Ato, there are other bHLH family proteins that are required for specification of sensory organs. *Achaete-Scute Complex* (*ASC*) bHLH genes are expressed with spatially regulated pattern to specify external sensory organ precursors (SOPs) (Cubas et al. 1991; Ghysen and Dambly-Chaudiere 1989; Romani et al. 1989; Skeath and Carroll 1991), and *amos* for multiple dendritic neurons in olfactory sense organs (Goulding et al. 2000; Huang et al. 2000). These proteins that are expressed in specific tissues are categorized as class II bHLH family transcription factors, in contrast to the class I bHLH factors expressed in a broader range of tissues. Tissue-specific class II bHLH proteins form heterodimeric complexes with class I bHLH proteins and directly bind to E-box consensus sequences of target genes through their basic domains.

Daughterless (Da), initially identified as an important factor for sex determination, is the only known neural class I bHLH protein in *Drosophila* (Caudy et al. 1988b). Like other class I proteins, Da has been thought to be expressed ubiquitously in a broad range of tissues and involved in diverse developmental processes

including neurogenesis, depending on its class II bHLH binding partners (Brown et al. 1996; Caudy et al. 1988a, b; Cronmiller et al. 1988). Since each tissue-specific class II bHLH protein requires Da to form a functional heterodimeric complex, both class I Da and class II bHLH proteins are important for their proneural function.

Interestingly, although Da protein is ubiquitously expressed in the eye disc, it is selectively upregulated in the morphogenetic furrow (Brown et al. 1996). Further analysis has revealed that there are two distinct patterns of Da upregulation in the furrow: a broad low-level upregulation and a stronger Da expression in the non-neural cells surrounding the R8 cells between proneural clusters (Fig. 5) (Lim et al. 2008). This pattern indicates that Da expression may be regulated in coordination with the process of neurogenesis in the furrow. Indeed, Da expression is dynamically regulated in the furrow by multiple mechanisms including Hh and Dpp signaling pathways.

Because Ato is known to form a dimer with the type I bHLH factor Da to function as an active transcription factor, the upregulation of Da in the non-neural cells

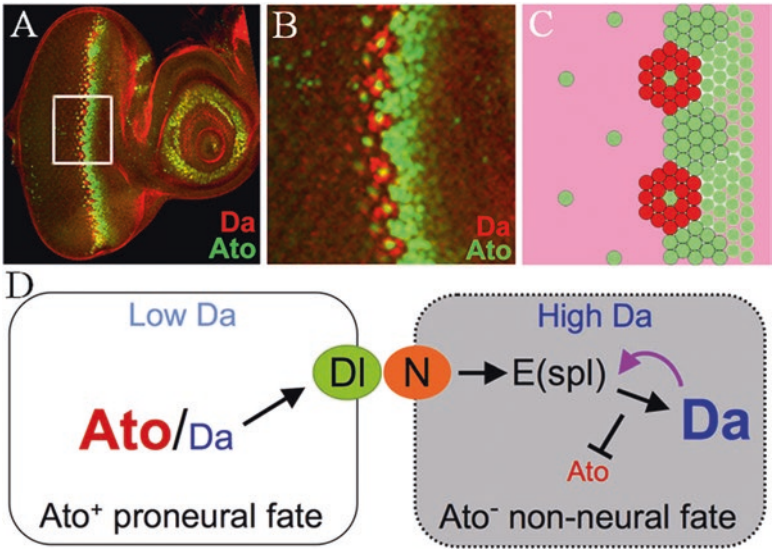

Fig. 5 Anti-proneural function of Daughterless. (**a**–**c**) Expression pattern of Da. Third instar eye disc stained with antibodies against Da and Ato. An area around the furrow (rectangle) in (**a**) is magnified in (**b**). (**c**) is a schematic of (**b**). In the furrow region, Da is expressed with a relatively low level in all Ato-expressing cells (green), but it is highly expressed in the cells surrounding singled-out Ato-positive R8 cells just behind the furrow (red). Outside the furrow, Da is expressed broadly at a low level anterior and posterior to the furrow region (pink). (**d**) A model for Da function during retinal neurogenesis. Da has dual functions as a proneural and an anti-proneural factor depending on expression level during early retinal neurogenesis. In Ato-positive proneural cells, a low level of Da forms a heterodimer with Ato to function as a proneural factor. In neighboring cells, Da is further upregulated by N-E(spl) pathway. A positive feedback regulation between E(spl) and Da represses Ato expression to antagonize neural specification. Adapted from Lim et al. (2008)

between proneural clusters is unexpected. Remarkably, loss of Da in the furrow leads to an expansion of Ato expression in mutant clones, indicating that Da acts as a negative factor for Ato expression. Despite the expanded Ato expression, there is no retinal differentiation within *da* LOF clones because Ato target genes like *sens* necessary for retinal differentiation are not induced in the absence of Da. In contrast, overexpression of Da results in the repression of Ato expression in the furrow. These results, together with the specific upregulation of Da between proneural groups, suggest that Da has both proneural and anti-proneural functions depending on the expression level and cell types in the furrow (Lim et al. 2008).

Because Da functions as a negative regulator of Ato expression in the furrow, it is an intriguing question whether this Da function is related to the lateral inhibition by N signaling. Notch-dependent lateral inhibition is mediated by E(spl), another bHLH family transcription factor. Clonal loss of Da in the region covering the furrow leads to a loss of E(spl) with concomitant upregulation of Ato. Moreover, ectopic expression of Da causes strong reduction of E(spl) expression. Interestingly, loss of N or E(spl) also results in reduction of Da expression. Thus, Da promotes Notch signaling to activate E(spl) expression, and both Da and Notch signaling cooperatively repress Ato expression to refine single R8 cell selection. In this model, a high level of Ato induced in the proneural group cells activates Dl, resulting in the Notch activation in the adjacent cells. Consequently, E(spl) expressed in these cells induces Da expression, resulting in a high-level Da expression and repression of Ato in these cells. The high-level Da in these cells also activates E(spl) expression by a feedback regulation, thus strengthening the difference between the proneural and non-neural cells (Fig. 5) (Lim et al. 2008). The dual function of Da was also found from a genetic screen for *ato* modifiers. One of the dominant enhancers turned out to be LOF alleles of *da*. LOF *da* clones showed expanded 3′ *ato-lacZ* in the furrow while decreasing 5′ *ato-lacZ* expression posterior to the furrow, consistent with the position-dependent dual function of Da in the regulation of *ato* (Melicharek et al. 2008).

Recent studies have also shown that an interaction of broadly expressed type I bHLH genes regulate tissue-specific cell fates. The Id family HLH proteins, called type V, do not have the basic domain so that the heterodimers of type II and V protein cannot function as transcription factors due to their inability to bind DNA. Extramacrochaete (Emc) is the only type V HLH protein in *Drosophila*. Although Emc is expressed broadly in most tissues, it is expressed at a low level in the furrow of an eye disc where Da expression is upregulated (Bhattacharya and Baker 2011; Brown et al. 1995). Da is expressed highly in the clones of LOF *emc* mutant cells. This indicates that Emc negatively regulates Da expression. However, loss of Da causes strong reduction of Emc, indicating that Da is required for Emc expression. Thus, Emc and Da appear to function in an unusual negative feedback loop where Da is necessary for the expression of its inhibitor Emc (Bhattacharya and Baker 2012).

It was proposed that such network of type I and V HLH proteins might be a general mechanism for the regulation of type II HLH expression in developmental decision makings during neurogenesis, not only in *Drosophila* but also in mammalian systems. As mentioned earlier, there are two levels of Da upregulation in the furrow: a general weak Da upregulation in the furrow and a selective stronger upregulation between the proneural groups in the furrow. In contrast to the high levels of Da in the furrow, Emc is low. Thus, it would be interesting to see whether Emc may be expressed dynamically within the furrow to cross-talk with different levels of Da in the furrow.

Concluding Remarks

Neural differentiation involves a series of inductive events to generate neurons from an undifferentiated epithelium. Neurogenesis is initiated in the morphogenetic furrow by secreted factors like Dpp and Hh as well as proneural transcription factors like Ato. Generation of ommatidial pattern in the developing eye is established by interaction of these positive factors and various negative regulators.

Several key negative factors involved in Ato repression and ommatidial spacing were discussed in this chapter. Notch is a major negative regulator of Ato expression and is essential for spatial patterning as well as fate specification of photoreceptor cells. Lateral inhibition by N may also be mediated by ligand-independent and cis-inhibition mechanisms.

Ato expression is also regulated negatively by Da. Da is an essential partner of Ato for its proneural function, but its high level around the proneural groups antagonizes Ato expression to generate interommatidial space. This anti-proneural function of Da is positively regulated by E(spl), a target of Notch signaling. Da and E(spl) form a feedback loop to promote their expression, which probably reinforces the lateral inhibition of Ato expression.

Bar homeodomain proteins are major negative regulator of Ato expression behind the furrow. Bar-expressing undifferentiated cells near the furrow can repress Ato expression, thus preventing ectopic formation of photoreceptors. Negative feedback regulation between Ato and Bar is important for ommatidial patterning. It would be interesting to see whether homologs for Ato and Bar have similar functional relationship in developing vertebrate eyes.

Acknowledgements I am thankful for Drs. Kyung Cho and Amit Singh for helpful comments. I apologize for many investigators whose work might not have been cited due to space limitation or my ignorance. This work was supported by grants from the World Class University Program (R31-2008-000-10071-0), National Research Laboratory grant (NRF-2011-0028326), and Global Research Laboratory grant (2014K1A1A2042982) through the National Research Foundation of Korea funded by the Ministry of Education, Science & Technology.

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Adhesion and the Cytoskeleton in the *Drosophila* Pupal Eye

Ruth I. Johnson

Introduction

The cells of the *Drosophila* eye are organized to generate a precise pattern during pupal development (Cagan and Ready 1989a; Wolff and Ready 1993). This process requires coordinated cell fate specification, cell growth and changes in cell shape, local cell movements and competition between cells for specific positions about an ommatidium, and apoptosis to remove surplus cells. Retinal cells occupy specific positions as they begin to differentiate in the pupa and they adopt easily discernable stereotypical shapes, so that patterning errors can be detected easily. This feature, coupled with the powerful genetic tools available in flies, and advances that allow us to visualize subcellular structures in the live eye make the pupal eye an excellent model to study the molecular mechanisms that integrate to orchestrate, drive, and regulate the morphogenesis of complex multicellular organs. The focus of this chapter is the role of adhesion receptors and the associated cytoskeleton in organizing the epithelial cells that surround and support the photoreceptors of the eye. These epithelial support/accessory cells are dynamically reorganized in the pupa and several recent studies that have begun to characterize the cytoskeleton and junctions in these cells will be discussed. The signal transduction pathways and transcription factors that drive photoreceptor and epithelial accessory cell recruitment and specification are discussed elsewhere in this book and in recent reviews (Charlton-Perkins and Cook 2010; Kumar 2012).

R. I. Johnson (✉)
Biology Department, Wesleyan University, Middletown, CT, USA
e-mail: rijohnson@wesleyan.edu

A. Singh, M. Kango-Singh (eds.), *Molecular Genetics of Axial Patterning, Growth and Disease in Drosophila Eye*,
https://doi.org/10.1007/978-3-030-42246-2_7

A Descriptive Overview of Eye Patterning

Around 750 unit eyes, called ommatidia, are neatly packed into a honeycomb array to form each *Drosophila* compound eye (Fig. 1a) (Wolff and Ready 1993). In the adult, this organization is evident in the hexagonal outlines of the domed corneal lenses that cap each ommatidium and focus light onto the underlying photoreceptor rhabdomeres positioned in the center of each ommatidium (Fig. 1b) (Charlton-Perkins et al. 2011). The photoreceptors of each ommatidium are surrounded by four translucent cone cells and two primary pigment cells (1°s) that together generate the lens material during late pupal development and must therefore be correctly organized for the lens to be correctly shaped (Cagan and Ready 1989a). The 1° pigment cells, together with the six secondary (2°) and three tertiary (3°) pigment cells that surround each ommatidium, provide mechanical support for the tall photoreceptor bundle and, in addition, generate pigment that optically isolates neighboring ommatidia and gives the eye its characteristic red color (Cagan and Ready 1989a; Shoup 1966; Waddington and Perry 1960; Wolff and Ready 1993).

To understand how this remarkable structure is formed, one must step back in time and examine the eye during its larval and pupal development. The organization of ommatidia begins in the late third larval instar eye disc (Fig. 2a–c) (Wolff and

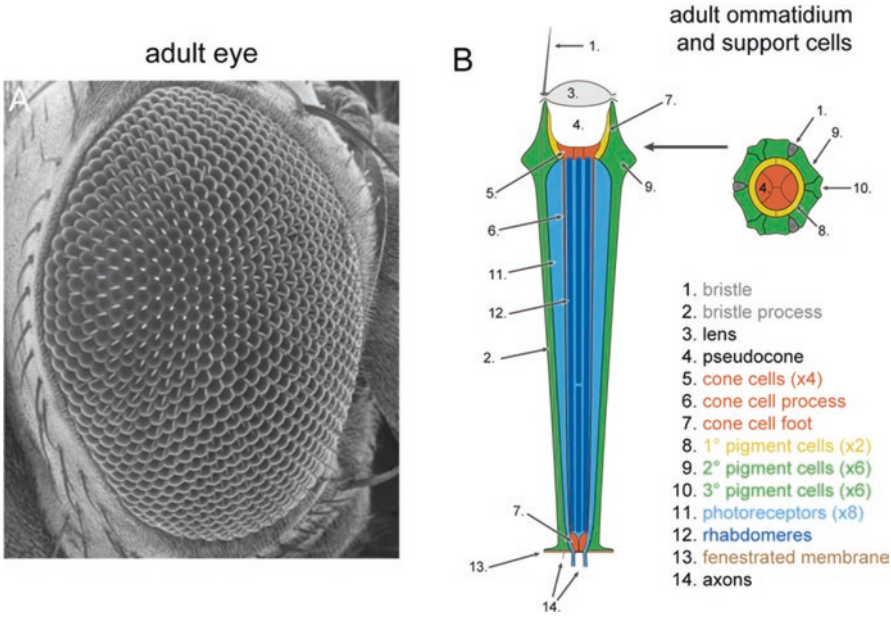

Fig. 1 Morphology of the adult eye. (**a**) Scanning electron micrograph of an adult eye, which contains around 750 ommatidia, each capped by a distinctive lens. Posterior is to the left. (**b**) Cartoon of a single adult ommatidium and its support cells. Image in **b** is adapted from Cagan and Ready (1989a)

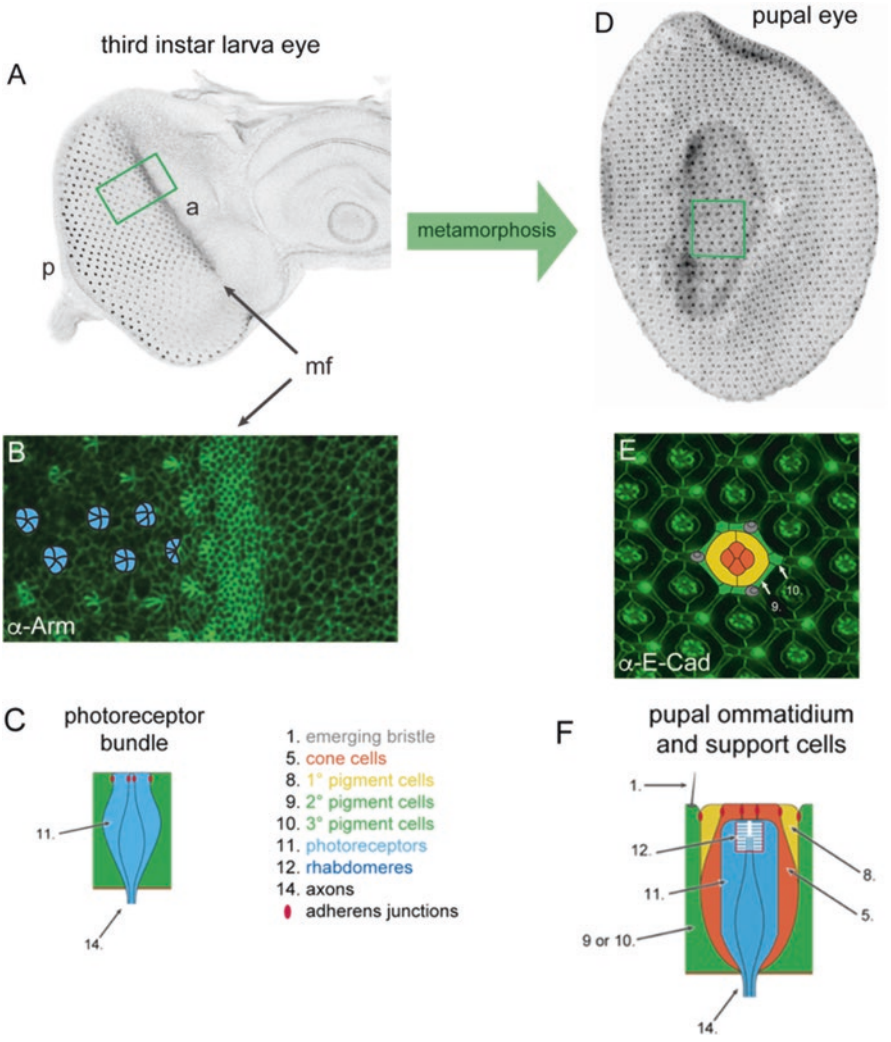

Fig. 2 The larval and pupal eye neuroepithelium. (**a**) An eye-antennal disc dissected from a wandering third instar larva. The green box approximately corresponds to (**b**), a small region of the eye field. The tissue has been incubated in antibodies to β-Catenin (Armadillo, Arm). Ommatidial clusters, illustrated in blue, emerge behind the morphogenetic furrow (mf) which travels from posterior (p) to anterior (a). (**c**) An illustration of a longitudinal view of a single larval photoreceptor cluster. Photoreceptors are in blue and surrounding undifferentiated epithelial cells in green. (**d**) A pupal eye dissected at 40 h after puparium formation (APF), incubated with antibodies to E-Cadherin (E-Cad). The green box corresponds approximately to the image presented in (**e**). The cone cells and 1° pigment cells of a single ommatidium, and its surrounding support cells have been colored according to the key provided. (**f**) An illustration of a longitudinal view of a pupal ommatidium and surrounding support cells. Cell types are listed in key. Illustrations in **c** and **d** are inspired by Longley and Ready (1995); Tepass and Harris (2007)

Ready 1991a) when sequential recruitment of photoreceptor precursor cells occurs as a wave across the eye disc and photoreceptor bundles that will form the core of each ommatidium emerge in staggered rows, surrounded by a sea of undifferentiated cells (Ready et al. 1976; Tomlinson and Ready 1987b). A subset of these undifferentiated cells then enter a final round of mitosis but, otherwise, cell proliferation halts (de Nooij and Hariharan 1995; Wolff and Ready 1991a). The eight photoreceptors adopt stereotypical positions within each cluster and four cone cells are then recruited to each ommatidium from the undifferentiated cell pool just before the organism begins to pupate (Cagan and Ready 1989a; Ready et al. 1976; Tomlinson 1988; Tomlinson and Ready 1987a). The eye undergoes dramatic morphogenesis in the early pupa to emerge inverted and cupping the optic lobe. Photoreceptor axons projecting from each ommatidium synapse at discrete layers of the medulla within the optic lobe, securing the eye to the optic lobe (Agi et al. 2014; Hakeda-Suzuki and Suzuki 2014). Within the eye, each photoreceptor bundle gradually becomes submerged below the apical surface of the tissue (Fig. 2e and f) as the photoreceptors bend ~90° to reorient their apical surfaces inward toward the core of each ommatidium (Cagan and Ready 1989a; Longley and Ready, 1995). These "inward-facing" photoreceptor membranes then elaborate to form the light-sensing rhabdomeres (Charlton-Perkins and Cook 2010; Knust 2007; Pichaud 2014).

Apart from the regular spacing of the fledgling ommatidia—which at this point are comprised of photoreceptors and cone cells—the eye neuroepithelium appears relatively disorganized in the very early pupa (Fig. 3a, at left). Next, two cells

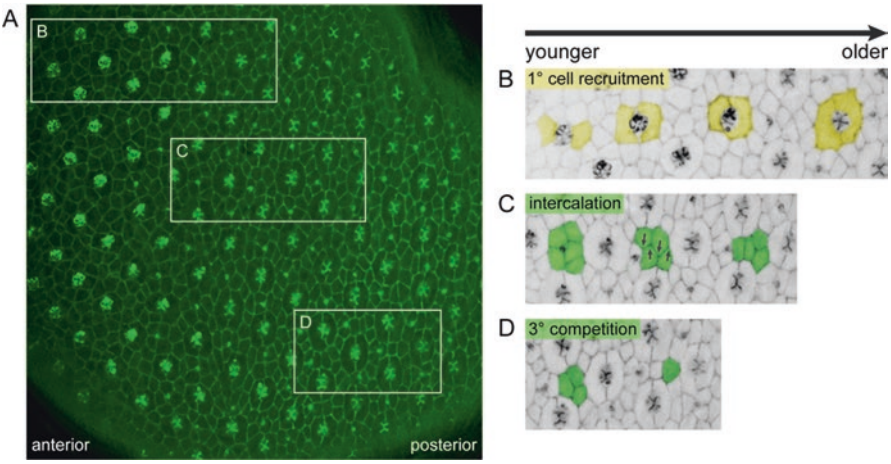

Fig. 3 Step-wise morphogenesis in the pupal eye. (**a**) A pupal eye at 20 h APF, marked by a gradient of development from anterior (left) to posterior (right). The adherens junctions are labelled in this eye with GFP-tagged E-Cad and GFP-tagged α-Cat. The boxed regions correspond to panels (**b**), which tracks the recruitment and morphogenesis of 1° cells, (**c**) intercalation of lattice cells and (**d**) competition of three cells for the 3° cell position. In keeping with the color-scheme of Figs. 1 and 2 1° cells are pseudo-colored yellow and lattice cells are green. Arrows in (**c**) indicate direction of cell intercalation. Images adapted from Hellerman et al. (2015)

immediately adjoining the anterior and posterior cone cells of each ommatidium are recruited to adopt the 1° cell fate and these rapidly stretch to encircle the four cone cells (Fig. 3b) (Cagan and Ready 1989b; Nagaraj and Banerjee 2007). At about the same time, three bristles emerge around each ommatidium (Cagan and Ready 1989a) and patterning of the lattice begins.

To generate the ordered arrangement of the honeycomb lattice, the remaining sea of interommatidial pigment precursor cells is reduced to a single-file lattice of cells via local cell movements and intercalation (Fig. 3c) (Larson et al. 2008). Lattice cells (this term will be used to refer to all interommatidial cells in this chapter, regardless of their state of differentiation) are now roughly organized into a hexagon around each ommatidium and the three cells closest to each vertex (not occupied by a bristle group) compete to acquire the corner position and hence the 3° cell fate (Fig. 3d) (Hellerman et al. 2015; Larson et al. 2008). Apoptosis gradually removes excess cells from the eye field (Bushnell et al. 2018; Cordero et al. 2004; Miller and Cagan 1998; Monserrate and Brachmann 2007; Wolff and Ready 1991b), leaving six to become the 2° cells that form the edges of the hexagon (Cagan and Ready 1989a). Over the next few hours, the central 1° cell pair of each ommatidium expands to adopt an almost-circular outline, the 2° cells narrow to become rectangular and the 3°s become more or less hexagonal (Fig. 2e). The result is a near-perfect honeycomb lattice and ommatidia that display little variation in their arrangement and shape (except close to the periphery of the eye). The adhesive junctions and cytoskeletal structures that contribute to the morphogenesis of this precise cone and pigment cell arrangement are the focus of this chapter. However, these will be discussed mainly from a two-dimensional perspective as we consider cell shapes and cytoskeletal organization as observed at the apical surface of the eye epithelium, simply because their three-dimensional morphologies have at this point been poorly explored.

Junctions and the Cytoskeleton in the Eye: The Basics

Several types of junctions connect epithelial cells to each other (Alberts et al. 2015): tight junctions seal neighboring cells together with a dense molecular mesh that restricts the paracellular diffusion of ions and molecules; adherens junctions and desmosomes mediate cell–cell adhesion that is strong enough to resist mechanical disruption when an epithelium or its cells are pushed, pulled, grow, or shrink; and basal focal adhesions anchor epithelial cells to extracellular matrix proteins. These epithelial cell junctions have similar structures (Anderson and Van Itallie 2009; Shapiro and Weis 2009; Wehrle-Haller 2012) in that (a) they contain transmembrane adhesion receptors whose extracellular domains bind similar adhesion receptors displayed by neighboring cells or, in the case of focal adhesions, extracellular matrix proteins; and (b) they contain cytoplasmic proteins that engage the cytoskeleton.

Over the past two decades, studies in vertebrate and *Drosophila* cell lines and tissues have transformed our understanding of the assembly, regulation, and function of adhesive junctions. Much attention has been paid to adherens junctions,

which accumulate along with an associated actin filament network in a band about the circumference of epithelial cells to form the zonula adherens. We now increasingly appreciate that the zonula adherens does not passively hold epithelial cells together but instead actively responds to and even generates mechanical forces and stimuli that contribute to embryo and tissue morphogenesis (Harris 2018; Heer and Martin 2017; Mao and Baum 2015; Roper 2015).

In *Drosophila*, the adherens junction is the most apical junction complex in epithelial cells (Muller 2000; Tepass et al. 2001) and the septate junctions, which are functionally analogous to vertebrate tight junctions, lie below adherens junctions (Furuse and Tsukita 2006). The fly eye is a pseudostratified columnar epithelium and detecting the core epithelial adherens junction receptor E-Cadherin (E-Cad, encoded by *shotgun* in *Drosophila*) that generates the transmembrane backbone of adherens junctions, or the catenins that associate with E-Cad's intracellular domain (p120-Catenin, α-Catenin, or β-Catenin which is encoded by *armadillo*) is a useful strategy for highlighting the apical shapes of retinal cells (Figs. 2 and 3). In addition, adhesion complexes are sparsely distributed along the lateral membranes of the pigment and cone cells (Pichaud 2014): the function of these lateral junctions has not been explored although it is plausible that they contribute to riveting retinal cells together in the absence of desmosomes, which are not found in insects.

E-Cad is expressed in all cells of the eye neuroepithelium but N-Cadherin (N-Cad) is found only in the photoreceptors and the central four cone cells (Fig. 4a) (Hayashi and Carthew 2004; Mirkovic and Mlodzik 2006). E-Cad and N-Cad are

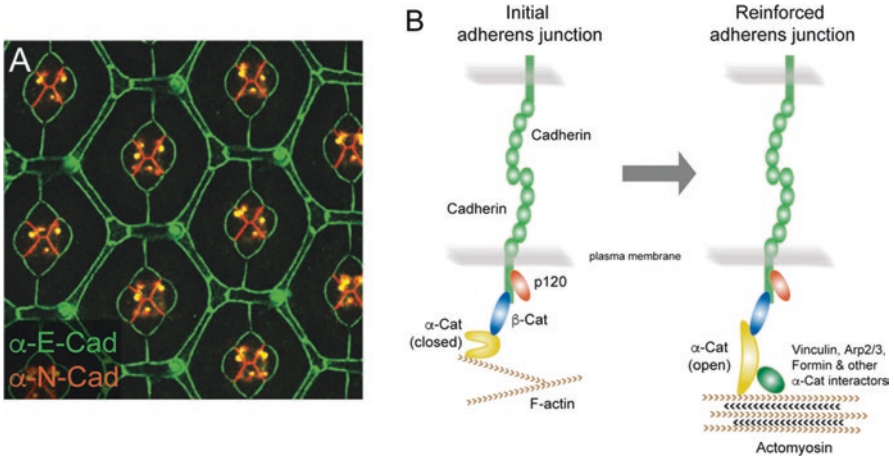

Fig. 4 Adherens junctions in the pupal eye. (**a**) E-Cad (green) and N-Cad (red) in the pupal eye. N-Cad is expressed in the four cone cells and therefore locates to adherens junctions between them. (**b**) Cartoon of a newly formed adherens junction (left) and adherens junction that has been reinforced in response to tension (right). The extracellular cadherin domains mediate interactions between the classical cadherins. α-Catenin that undergoes a conformational modification in response to force, revealing binding sites for a variety of proteins including those that activate F-actin polymerization. Image in **a** adapted from Chan et al. (2017). Illustrations in **b** are inspired by Charras and Yap (2018)

classical cadherins that interact homophilically across the intercellular space using their characteristic extracellular cadherin domains (Fig. 4a) (Ishiyama and Ikura 2012; Nose et al. 1990; Takeichi 2014; Tomschy et al. 1996) while p120-Catenin and β-Catenin interact with their intracellular domains (Huber et al. 2001; Huber and Weis 2001; Ishiyama et al. 2010; McCrea and Gumbiner 1991; McCrea et al. 1991; Thoreson et al. 2000). Classical cadherins connect to the cytoskeleton via α-Catenin, which binds both F-actin and β-Catenin (Aberle et al. 1994; Ozawa et al. 1990; Rimm et al. 1995).

In epithelial tissues, the cytoskeleton is directly affected by changes in adherens junctions complexes. In large part, this is due to recruitment of actin regulatory proteins to adherens junctions or the associated cytoskeleton including Arp2/3, which is activated to promote actin branching at newly formed cadherin junctions (Kovacs et al. 2002); Formins, which nucleate the formation of F-actin bundles, like those associated with typical zonula adherens (Acharya et al. 2017; Grikscheit et al. 2015; Kobielak et al. 2004; Rao and Zaidel-Bar 2016); and non-muscle myosin II (Myo-II), which when activated introduces tensile forces into a cell that trigger conformational changes in α-Catenin enabling recruitment of Vinculin and additional actin regulators including Arp2/3, Vasp, and the Formins to the adherens junction (Bertocchi et al. 2017; Brindle et al. 1996; Choi et al. 2012; DeMali et al. 2002; Kim et al. 2015; Tang and Brieher 2012; Yao et al. 2014; Yonemura et al. 2010). Hence, the introduction of tensile force into a cell can promote junctional actin and myosin remodeling or accumulation which can trigger additional cadherin recruitment, to fortify adhesion (Leerberg et al. 2014; Liu et al. 2010; Maitre et al. 2012; Scott et al. 2006; Thomas et al. 2013). As such, the adherens junction is therefore often described as a mechanosensor that detects and responds to forces (Charras and Yap 2018; Pinheiro and Bellaiche 2018), but how this property is utilized to control *Drosophila* eye morphogenesis is not yet known.

Using phalloidin to detect F-actin in the early mainly unpatterned pupal eye reveals faint accumulation of actin at adherens junctions of all cells and what might be branched actin structures associating with these junctions (Fig. 6a) (Johnson et al. 2008). These structures are reminiscent of branched actin and hence could reflect activation of Arp2/3 at newly formed cadherin junctions, as reported elsewhere (Kovacs et al. 2002) or at junctions undergoing remodeling. The accumulation of actin in a zonula adherens becomes more pronounced as the tissue becomes ordered, suggesting increasing fortification of adhesion that might, at least in part, be in response to tension and other biophysical parameters associated with the distinctive cell shapes observed in the eye (Fig. 6b and c, cortical and medial actin bundles also become more pronounced). Understanding how the cytoskeleton and junctions respond to or transmit force to ensure correct eye patterning is an interesting topic for future research.

Several non-cadherin adhesion molecules are also crucial for eye patterning, including the *Drosophila* orthologs of the Nephrin/Neph proteins, Roughest (Rst), Hibris (Hbs), Sticks and Stones (SNS), and Kin of irre (Kirre), which are collectively called the irre cell recognition module (IRM) proteins. These contain extracellular immunoglobulin-repeat domains that mediate their *trans* heterophilic

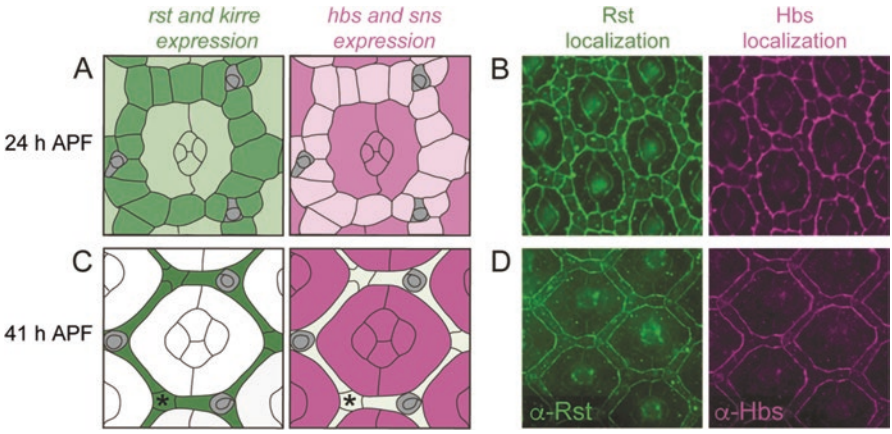

Fig. 5 The IRM proteins in the pupal eye. (**a**) Illustrations of the expression patterns of *rst* and *kirre* (left) and *hbs* and *sns* (right) in the eye at 24 h APF. The depth of color represents relative expression of these transcripts in the ommatidia or lattice cells. (**b**) Because Rst and Hbs form heterophilic complexes, they accumulate at lattice cell: 1° cell borders. Some complexes are also detected at boundaries between lattice cells due to residual expression of *rst* and *hbs*. Localization of Kirre/Sns complexes is similar (not shown). (**c**) Expression of *rst* and *kirre* becomes limited to lattice cells and that of *hbs* and *sns* to the ommatidia, so that (**d**) complexes of these proteins are detected almost exclusively to lattice cell: 1° cell boundaries. Figure adapted from Johnson et al. (2012)

interactions (Fischbach et al. 2009) and the expression patterns of partner IRM proteins (Rst binds Hbs, and Sns binds Kirre) evolve into complementary domains in the pupal eye (Fig. 5) (Bao and Cagan 2005; Bao et al. 2010; Grzeschik and Knust 2005; Johnson et al. 2012; Reiter et al. 1996; Tanenbaum et al. 2000). In other words, while *rst*, *hbs*, *sns,* and *kirre* are expressed in almost all cells of the early retina (Fig. 5a), *rst* and *kirre* expression is then removed from 1° cells and *hbs* and *sns* expression is removed from the lattice cells. The result is complementary expression of IRM partners in adjoining cells (Fig. 5c) and accumulation of Rst/Hbs and SNS/Kirre complexes at adherens junctions bordering their expression domains (Fig. 5b and d). These complexes are essential for correct pupal eye morphogenesis, as discussed further below.

The Arrangement and Shaping of Cone Cells

Because N-Cad is expressed in the four cone cells and not the surrounding 1°s (Hayashi and Carthew 2004; Nern et al. 2005), homophilic N-cad adherens junctions locate specifically to boundaries between cone cells (Fig. 4a) (Hayashi and Carthew 2004). These adherens junctions are crucial for cone cells to adopt their stereotypical arrangement at the center of each ommatidium because the differential expression of *N-* and *E-Cadherin* manifests in different adhesion properties of the

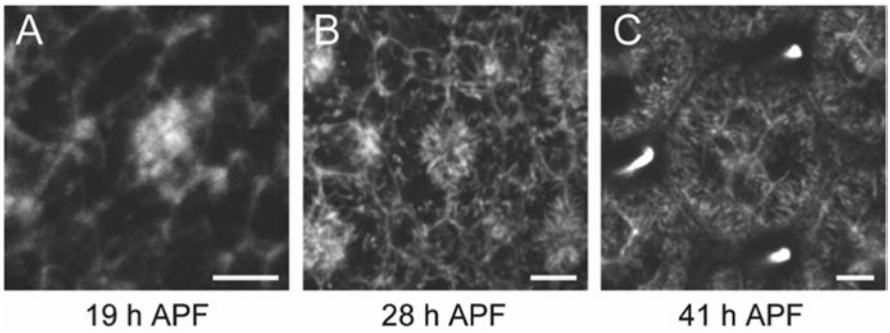

Fig. 6 Phalloidin staining of the eye reveals a complex cytoskeleton. Small regions of retinas incubated in phalloidin at (**a**) 19 h APF, (**b**) 28 h APF, and (**c**) 41 h APF. A single ommatidium is at the center of each image. Scale bars represent 5 μm. Images from Johnson et al. (2008)

cone and 1°cells. Higher adhesion between cone cells segregates them from the 1°s, positions them in the center of the ommatidium, and contributes to the relatively straight geometries of the membranes between cone cells and the rounded shape of the cone cell: 1° cell interfaces (Hayashi and Carthew 2004). These properties exemplify the "differential adhesion hypothesis" (Steinberg 1963) demonstrated in vitro when cells with different adhesion strengths are co-cultured: cells with stronger adhesion aggregate together, are enveloped by cells with weaker adhesion properties, and the surface tension at the periphery of the more adherent aggregate is high (Foty and Steinberg 2005).

The shapes and arrangement of mature cone cells are distinctive, with dorsal and ventral cone cells in direct contact and positioned between the anterior and posterior cone cells (Fig. 7a). Mathematical models predict that strong homophilic N-Cad adhesion maximally expands cone cell: cone cell interfaces and constrains the remaining membrane that is shaped to minimize surface area (Hilgenfeldt et al. 2008; Kafer et al. 2007). This energetically favorable, adhesion-mediated cone cell arrangement is similar to the behavior of groups of soap bubbles, in which the bubble surfaces are minimized to reduce their contact with surrounding water molecules while contact between neighboring bubbles is maximized where the bubble membranes fuse (Fig. 7b). This soap bubble analogy was confirmed by genetically manipulating the number of cone cells in an ommatidium: the cone cells were always arranged in configurations that matched the arrangement of a similar number of soap bubbles (Fig. 7b, c) (Hayashi and Carthew 2004). Further, removing N-Cad from one (or two) cone cells left the remaining three (or two) wild-type cone cells grouped together in a configuration resembling that of the same number of soap bubbles (Fig. 7d). These genetic manipulations also reduced the junctional interfaces between mutant and wild-type cone cells while the interface between the *N-Cad* mutant cone cell(s) and neighboring 1° cell(s) expanded (Fig. 7d) (Hayashi and Carthew 2004). Thus the adhesion between cone cells (reflected by the length of junctions between them) influences the shape of the remaining apical circumference of the cell, which is of course constrained by the amount of available membrane.

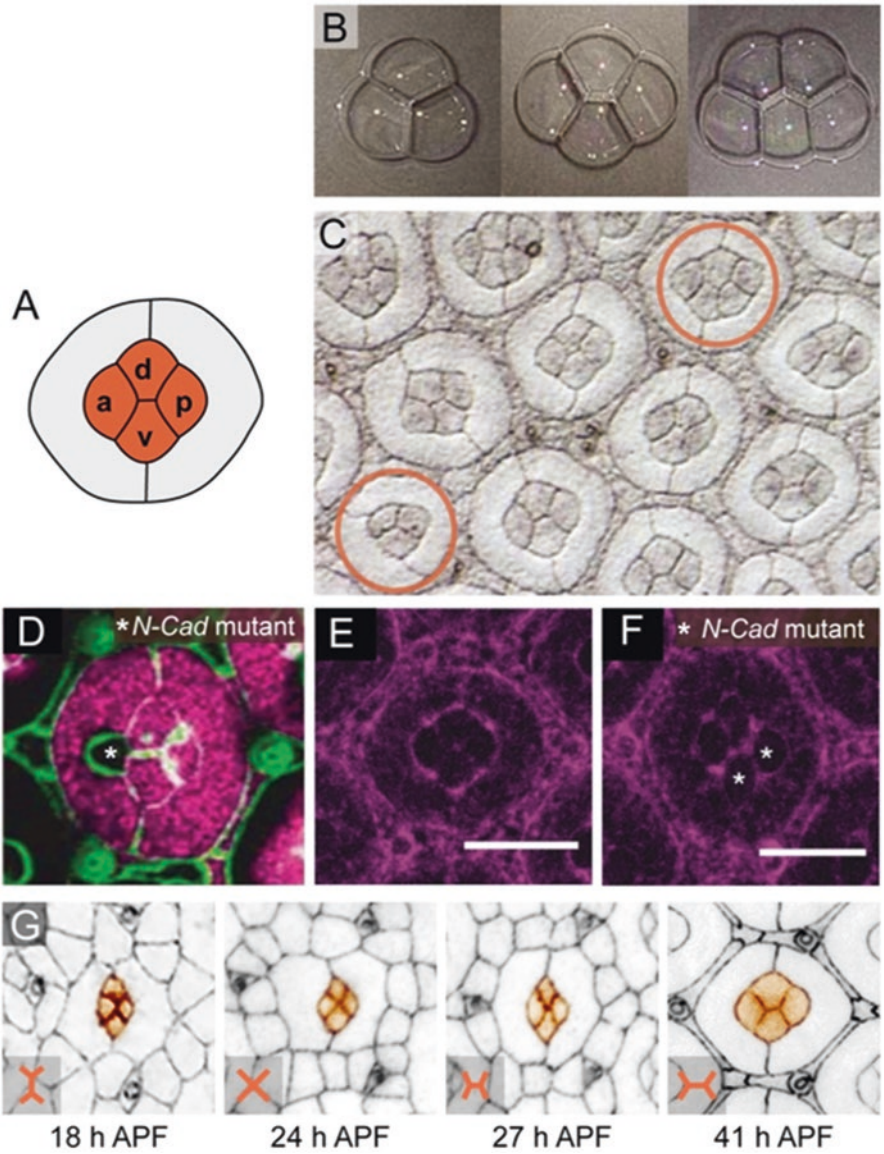

Fig. 7 Correct patterning of cone cells resembles the aggregation of four soap bubbles. (**a**) Cartoon of an ommatidium with the dorsal (d), ventral (v), anterior (a), and posterior (p) cone cells labelled. (**b**) Soap bubbles arrange and fuse in energetically favorable configurations that minimize their surface areas. (**c**) The arrangement of cone cells mimics that of soap bubbles. In this mutant retina, the number of cone cells per ommatidium is sometimes incorrect: examples of ommatidia with three and five cone cells are circled. (**d**) When *N-Cad* expression was removed from one cone cell (not magenta, labelled with an asterisk), the shape of that cone cell changed and the remaining three cone cells aggregated just like three soap bubbles. (**e** and **f**) Myo-II is shown in magenta, scale bars represent 10 μm. In (**f**) the two cone cells Fig. 7 (continued) labelled with asterisks lack N-Cad: this alters the distribution of Myo-II so that it accumulates at the boundaries between *N-Cad* mutant and wild-type cone cells, altering cone cell arrangement. (**g**) The configuration of the cone cells undergoes a typical T1–T2–T3 transition, bringing the dorsal and ventral cone cells into contact. Cone cells are pseudo-colored orange and a cartoon depicting the cone cell boundaries is provided in each panel. Images **b**, **c**, and **d** adapted from Hayashi and Carthew (2004), **e** and **f** adapted from Chan et al. (2017)

Subsequent computational modeling predicted that the cortical tension at cone cell: cone cell junctions is lower than that at cone cell: 1° cell junctions (Kafer et al. 2007). Differences in actomyosin accumulation have now been shown to mediate these differences in cortical tension (Chan et al. 2017): Myo-II is detected at higher levels at cone cell: 1° cell junctions than at cone cell: cone cell boundaries (Fig. 7e) (this observation was also reported by Aigouy and Le Bivic, 2016). This suggests different regulation of Myo-II at interfaces between cells that do, and do not, have N-Cad and accordingly, in mosaic ommatidia, Myo-II accumulation increased at boundaries between *N-Cad* mutant and non-mutant cone cells. Chan et al. (2017) also provided experimental evidence suggesting that "free" N-Cad molecules located at cone cell: 1° cell boundaries but not incorporated into adherens junctions promote Myo-II accumulation at these locations. In addition, in mosaic ommatidia, more Myo-II accumulated at junctions between two *N-Cad* mutant cone cells (Fig. 7f), suggesting that myosin accumulation may be antagonized downstream of adherens junctions characterized by N-Cad. Hence, we conclude that cone cell morphologies are determined by N-Cad-mediated adhesion and Myo-II-mediated tension, which in turn is regulated by bound and free N-Cad, respectively.

In addition to N-Cad, other adhesion receptors expressed in cone cells (or the neighboring 1°s) can contribute to the final arrangements and shapes of cone cells. These include the IRM proteins as errors in the final arrangements of cone cells are frequently observed when *hbs*, *rst*, *sns,* or *kirre* expression is reduced during pupal eye patterning (Bao and Cagan 2005; Bao et al. 2010; Grillo-Hill and Wolff 2009). The contribution of these IRM complexes to the adhesion, cytoskeleton, and biophysical properties of cone cells remains to be explored.

But the story is more complex. In early pupal stages, the anterior and posterior cone cells, rather than the dorsal and ventral cones, are in direct contact (Fig. 7g). This configuration probably arises because the anterior and posterior cone cells are recruited to the ommatidium first, which leads to their expressing *N-Cad* first, leading to higher levels of N-Cad in the anterior and posterior cone cells that mediates their initial adhesion to each other (Cagan and Ready 1989a; Nagaraj and Banerjee 2007; Ready et al. 1976; Tomlinson 1988; Tomlinson and Ready 1987a). However, the cone cell quartet then undergoes morphological changes of a classic T1–T2–T3 junction exchange (Fig. 7g) (Bertet et al. 2004; Harris 2018), leaving the equatorial and polar cone cells adjoined. What drives this T1–T2–T3 transition in cone cells has not yet been studied. However, based on studies in other tissues, it is plausible that the cone cells are reorganized into the T1 conformation (Fig. 7g, second panel) via polarized myosin activation at the adherens junction between anterior and posterior cone cells: this would generate a contractile force that shortens the anterior-posterior cone cell interface (Blankenship et al. 2006; Kasza et al. 2014; Simoes Sde et al. 2014). At the same time, contraction of an apical-medial actomyosin network in the anterior and posterior cone cells could "tug" on the contracting adherens junction to compromise junction stability and promote its rapid shortening (Levayer and Lecuit 2013; Rauzi et al. 2010; Tamada et al. 2012; Warrington et al. 2013). To then mediate transition of the cone cells into the T3 conformation (Fig. 7g, third panel), adhesion between the dorsal and ventral cone cells might be promoted by polarized accumulation of factors that favor adherens junction formation, including Par3 (Bazooka in *Drosophila*) and PTEN (Bardet et al. 2013; Simoes Sde et al. 2010). Elongation of the nascent dorsal-

ventral adherens junction might be enhanced by simultaneous contraction of the apical-medial actomyosin network in the anterior and posterior cone cells to gently "pull" on the expanding dorsal-ventral cone cell interface (Collinet et al. 2015; Yu and Fernandez-Gonzalez 2016). Whether these events—which have been nicely documented in the *Drosophila* embryo, for example—indeed do drive the T1–T2–T3 transition of cone cells remains to be confirmed, but perhaps a more interesting question will be: what mechanisms contribute to the timing of the T1–T2–T3 transition?

The Primary Pigment Cell Pair

Two cells about each ommatidium are recruited to become 1° cells in a process that is dependent on Notch signaling, which is activated by Delta expressed in cone cells (Cagan and Ready 1989b; Nagaraj and Banerjee 2007). The 1°: 1° junction forms and expands rapidly as soon as 1° cell pairs make contact when encircling the cone cells (Fig. 8a). This is essential: if the cone cells are not rapidly surrounded by 1°s

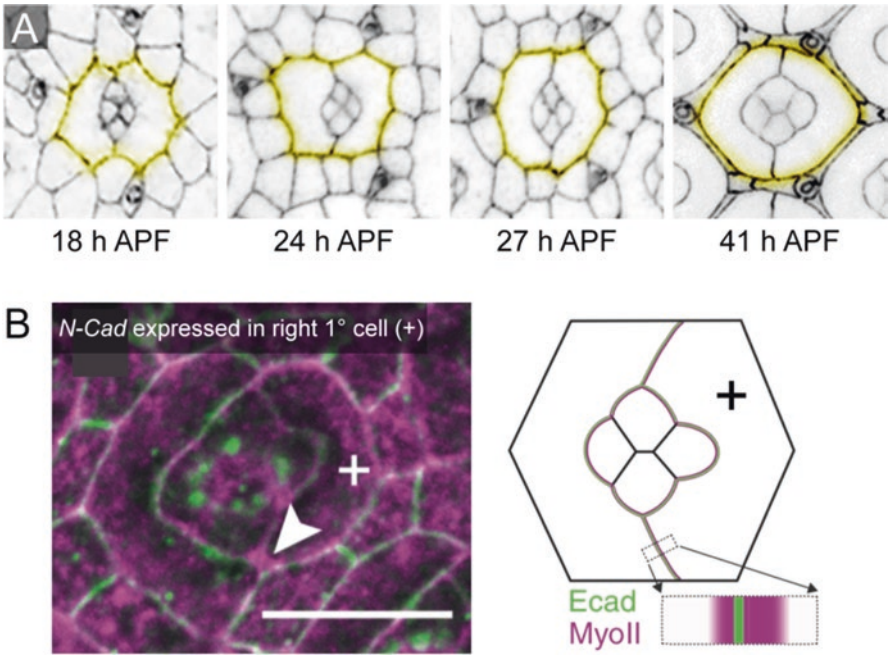

Fig. 8 Patterning of 1° cells. (**a**) The primary cell pair, outlined in yellow, encircle the cone cells. The lattice cell: 1° cell boundary is initially scalloped but becomes smooth and the 1°s acquire their eventual shape. The junctions between the two 1° cells are straight. (**b**) Ectopic expression of *N-Cad* in the right 1° cell (+) leads to accumulation of Myo-II (magenta) and constriction of that cell. Arrowhead indicates accumulation of Myo-II at the 1°:1° boundary, which is narrowed. Cartoon on left illustrates this ommatidium with relative accumulation of Myo-II on either side of the 1°:1° boundary. Data in **b** adapted from Chan et al. (2017)

but instead remain in contact with the undifferentiated lattice cells, these will be recruited to form additional 1°s as they are competent to respond to Delta (Cagan and Ready 1989b). The adherens junctions that bind neighboring 1° cells together are marked by localization of the IRM proteins (Figs. 4a and 5b). These junctions are straight and the 1°s approximately equal in size, suggesting that each 1° receives and emits similar uniform mechanical forces at this junction (Fig. 8a). Indeed, if Myo-II is unequally activated in one 1° cell during pupal development (in Fig. 8b this was achieved via ectopic *N-Cad* which led to additional Myo-II activity), then that 1° cell becomes smaller and the 1°: 1° cell interface is bent (Fig. 8b) (Chan et al. 2017; Gemp et al. 2011).

The 1° cells initially have scalloped outlines, with the vertex of each scallop projecting between pairs of lattice cells, but scalloping is reduced as the eye matures (Fig. 8a). Several mechanisms contribute to scalloping and its resolution. Firstly, IRM complexes (Hbs—Rst, and Sns—Kirre) preferentially localize to 1° cell: lattice cell adherens junctions (Fig. 5) and when expression of these proteins is experimentally increased, so is scalloping, suggesting that IRM complexes promote the extension of junctions between 1° and lattice cells to promote scalloping (Bao and Cagan 2005; Bao et al. 2010). Second, and as discussed later in this chapter, Myo-II accumulation at lattice cell: lattice cell borders promotes their contraction (Del Signore et al. 2018) which, presumably, augments scalloping of 1°s. Finally, it is plausible that differences in the contraction of actomyosin networks running parallel to 1°: lattice cell boundaries promote scalloping and its subsequent eradication as the 1° cells acquire their rounded final shape. The organization and activation of such actomyosin networks at abutting 1°: lattice cell boundaries has not yet been documented and these ideas have not been tested. But in the meantime, a recent study by Aigouy and Le Bivic (2016) surveyed the distribution of myosin in mature rounded 1°s: Myo-II accumulated along the entire concave surface of 1°s at 1°: lattice cell interfaces, as one would expect if actomyosin contraction was crucial for generating or maintaining the rounded 1° shape. An additional key component in this system might be the fly ortholog of ZO-1, Polychaetoid (Pyd). Vertebrate ZO-1 was recently shown to antagonize junctional localization of the myosin activators Shroom and ROCK, and to therefore antagonize the contraction of junction-associated actomyosin (Choi et al. 2016). Since *pyd* is expressed at higher levels in the lattice cells than in 1°s (Seppa et al. 2008), we might therefore predict that Pyd antagonizes actomyosin contraction in lattice cells as scalloping of 1°s is eradicated. According to this model, global overexpression of *pyd* or pyd^{RNAi} transgenes might eradicate the imbalance of actomyosin activity in neighboring cell populations. Indeed, when such experiments were performed, the lattice cell: 1° cell boundaries were straight, suggestive of equal tension within both cell populations (Seppa et al. 2008). Furthermore, when pyd^{RNAi} was expressed in only one of a 1° cell pair, the scalloping in that cell was pronounced (Seppa et al. 2008), as the model would predict.

Interestingly, in mature rounded 1° cells, examined when all trace of scalloping has been resolved, ordered actin bundles can be observed running perpendicular to the 1° cell: lattice cell borders, tiling across the width of the 1°s (Fig. 6c) (Johnson

et al. 2008). The role of these actin structures in shaping 1°s or maintaining their rounded structures deserves attention.

Rearrangement of Lattice Cells

Once the 1°s have encircled the cone cells, the apical volume of the ommatidium begins to increase and the lattice cells intercalate, bringing them into single file about each ommatidium (Fig. 3b) (Hellerman et al. 2015; Johnson et al. 2011; Larson et al. 2010). Although detailed analyses of actin cytoskeleton activity in intercalating lattice cells have not yet been done, several actin regulatory proteins are known to be required for lattice cell intercalation. These include the small GTPase Arf6 that is required for a lattice cell to generate a single, large protrusion that projects toward a target 1° cell (Johnson et al. 2011). In genetic experiments where *Arf6* expression was reduced, lattice cells generated multiple small protrusions that were frequently retracted, and intercalation was delayed or unsuccessful (Johnson et al. 2011). Arf6 has been demonstrated to promote Arp2/3 activity and actin filament growth in several *in vitro* studies and our current understanding is that Arf6 indirectly activates Rac1 which in turn activates the WAVE regulatory complex to promote Arp2/3 complex activity (D'Souza-Schorey and Chavrier 2006; Hu et al. 2009; Humphreys et al. 2013; Koo et al. 2007). Hence it is plausible that the same pathway is engaged in the pupal eye to drive the actin polymerization required for a cell to push between its neighbors and intercalate (Fig. 9a).

However, Arf6 activity must be polarized for a lattice cell to generate a single protrusion that correctly projects in the appropriate direction toward a target 1° (Fig. 9). Polarized localization of Arf6 inactivators (ArfGAPs including ASAP and ArfGAP3) at boundaries between lattice and 1° cells or preferential localization of Arf6 activators (including Siz and Psd) elsewhere in the cell could induce such polarized Arf6 activity (Johnson et al. 2011). Indeed, ASAP and ArfGAP3 are probably localized to lattice: 1° cell junctions via their interaction with Cindr, an adaptor protein that also interacts with the IRM proteins that are concentrated at this cell interface (Johnson et al. 2011). Furthermore, Cindr interacts with the actin capping proteins and like its orthologs probably has intrinsic F-actin capping function, so that the actin cytoskeleton at lattice: 1° cell junctions is stabilized during lattice cell intercalation (Fig. 9) (Bruck et al. 2006; Johnson et al. 2008, 2012; Tang and Brieher 2012). Hence a Cindr-ArfGAP-Arf6 pathway is proposed to repress actin activity where lattice cells bind 1°s, while unhindered Arf6 activity elsewhere in the cell promotes the formation of cellular protrusions that drive lattice cell intercalation (Fig. 9; direct spatial analysis of Arf6 activity, for example, is required for confirmation of this model). Other mechanisms that likely contribute to robust intercalation of lattice cells may include mechanisms shown to drive intercalation of cells in epithelial sheets, including polarized remodeling of the junctions between intercalating cells and polarized myosin activity (Harris 2018; Heer and Martin 2017; Pinheiro and Bellaiche 2018; Roper 2015).

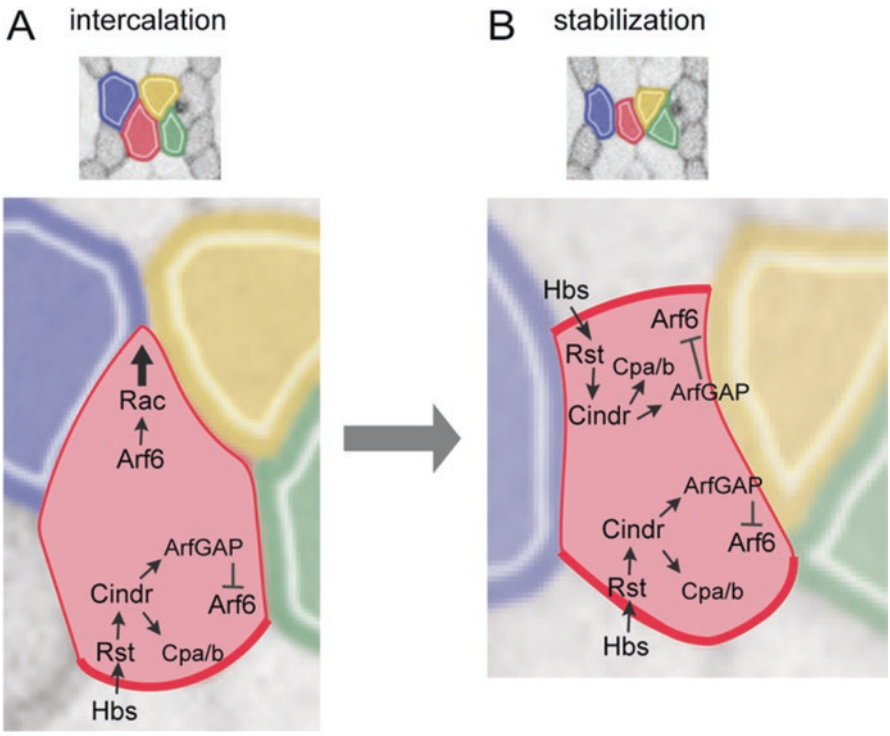

Fig. 9 Intercalation of lattice cells. (**a**) Model of the molecular interactions that cause cells to generate a projection in the direction of an opposite ommatidium. Arf6 and Rac1 activities are polarized. At boundaries between lattice and 1° cells, Arf6 activity is inhibited downstream of IRM complexes so that actin polymerization is spatially restricted. In addition, the activity of capping proteins (Cpa/b) is promoted at lattice: 1° cell junctions. (**b**) Once the lattice cell reaches its target ommatidium, rapid accumulation of IRM complexes antagonizes Arf6 at this new lattice cell: 1° cell boundary. (Adapted from Hellerman et al. 2015; Johnson et al. 2008, 2011)

The formation of stable adhesion complexes is key for lattice cells to be secured in single file after intercalating. When a lattice cell reaches its target 1° cell, a junction between the cells rapidly extends laterally (Hellerman et al. 2015). Mechanisms that drive this lateral extension have not been explored, although they likely include mechanisms that promote E-Cad recruitment and inhibit its endocytosis (Baum and Georgiou 2011; Kowalczyk and Nanes 2012; Takeichi 2014). The formation of IRM complexes is dependent on the formation of adherens junctions (Grzeschik and Knust 2005) and since IRM complexes accumulate at 1° cell: lattice cell junctions, it is possible that subsequent recruitment of Cindr and ArfGAPs then quietens Arf6 and Rac1 activity here, favoring junction stability (Fig. 9) (Johnson et al. 2011). Analyses of cell intercalation in live *rst* mutant retinas supports this model: *rst* mutant lattice cells struggle to establish or maintain their adhesion to 1° cells and lattice cells that have no contact with 1°s fail to move in a polarized manner toward an appropriate target 1° (Larson et al. 2008). These data also confirm that direct

contact between lattice and 1° cells is required for lattice cells to correctly interpret the polarity of the retina and move in the correct orientation. Unsurprisingly then, disrupting the expression of any of the IRM proteins leaves multiple rows of lattice cells around an ommatidium (Bao and Cagan 2005; Bao et al. 2010; Grzeschik and Knust 2005; Larson et al. 2008).

The battle for the corner 3° cell position begins as lattice cells intercalate (Fig. 3b) (Hellerman et al. 2015; Larson et al. 2008). This battle is usually between three cells. Each is already adhered to two ommatidia and each attempts to push toward the third, opposite ommatidium. After some buffeting, one cell eventually manages to project between the other two, reach its target ommatidium, and secure its position as the 3° cell (Hellerman et al. 2015). It is possible that the mechanisms that drive the oriented movement of lattice cell intercalation also drive the establishment of the 3° cell.

Shaping Lattice Cells

Following intercalation and the establishment of the 3° cell niche, excess cells—usually those located adjacent to bristle groups—are removed via apoptosis (Bushnell et al. 2018; Cordero et al. 2004; Miller and Cagan 1998; Monserrate and Brachmann 2007; Wolff and Ready 1991b). This leaves one cell along each edge of an ommatidium to adopt the characteristic rectangular 2° cell shape (Fig. 10a). At the same time, the apical area of each 3° shrinks and their hexagonal shapes become more regular (Fig. 10a). These shape changes transform the lattice into a near-perfect honeycomb.

Recent work examining the morphogenesis of lattice cells found that the adherens junctions between neighboring lattice cells repeatedly extend and contract in length as lattice cell shape is refined (Fig. 10b) (Del Signore et al. 2018). Extension of these junctions is facilitated by Arp2/3-dependent polymerization of the actin network while Myo-II accumulation drives contraction. Accordingly, increased localization of Rac1, PI(3,4,5)P_3 and WRC components, which activate the Arp2/3 complex (Hall 2005; Pollitt and Insall 2009), correlated with accumulation of actin

Fig. 10 (continued) secs later (t = 32, right panel) the boundary has extended and is now marked by accumulation of F-actin. (**c**) Model (left) of accumulation of Myo-II in 3°s and surrounding 2°s and resultant elongating or constricting forces. When myosin activity is removed in 2° cells (right, top panel, mutant cells labelled with GFP) or 3° cells (right, bottom panel), cell shapes and areas are modified (compare arrows with arrowheads). (**d**) Model (left) of F-actin accumulation in 3°s and surrounding 2°s and resultant elongating or constricting forces. When F-actin polymerization was disabled in 2° cells (top panel, mutant cells labelled with GFP) or 3° cells (bottom panel), the apical area of the cell was reduced (compare arrows with arrowheads). (**e**) Ectopic expression of *rst* in a single lattice cell caused that cell to maximize its adhesion to neighboring 1°s and expand. Panels on right show one of these cells at higher magnification. Data in **b**, **c**, and **d** adapted from Del Signore et al. (2018). Panel **f** adapted from Bao and Cagan (2005)

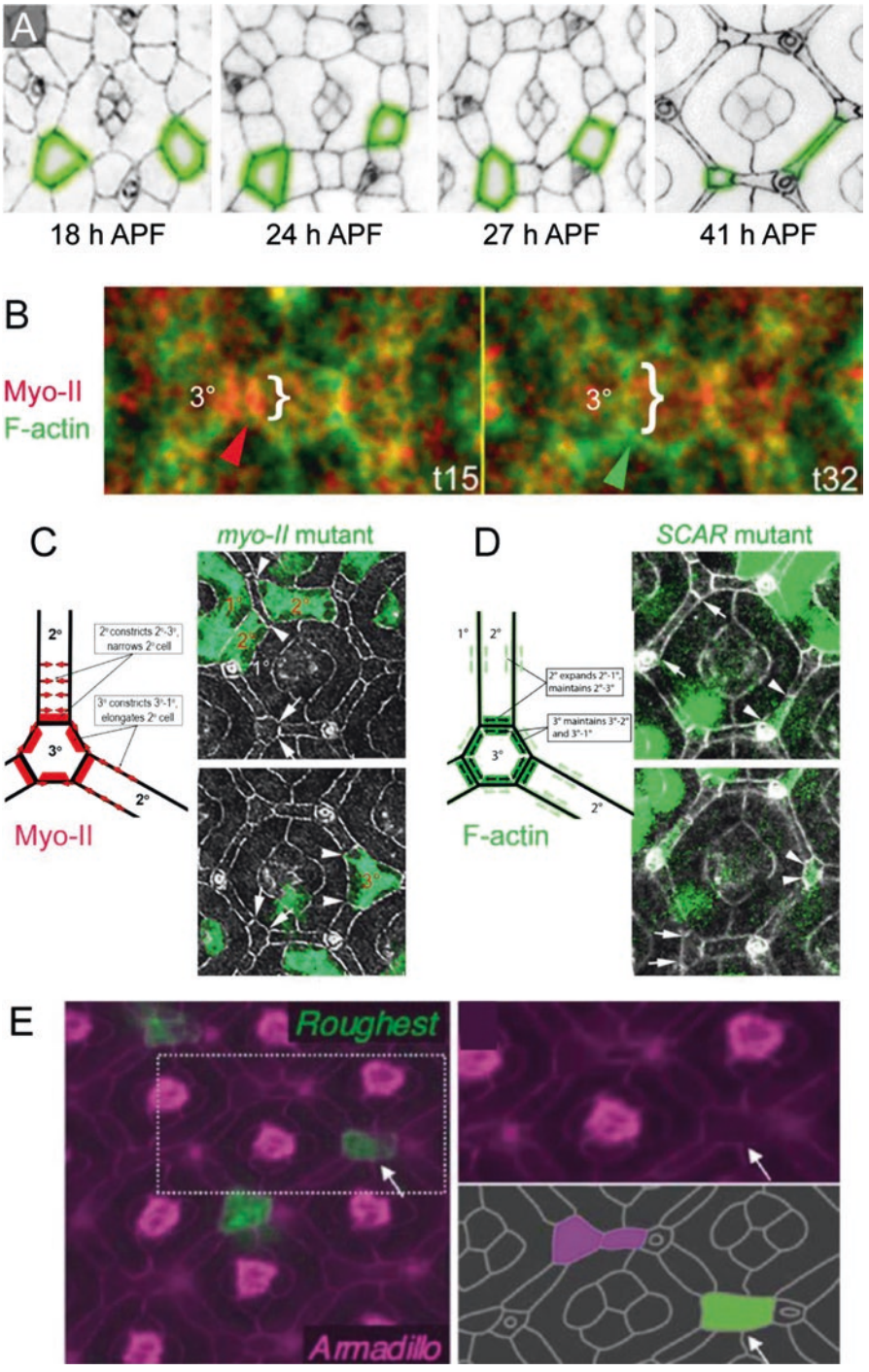

Fig. 10 Shaping the 2° and 3° cells. (**a**) Characteristic shapes of the 2° and 3° cells change over time. Examples of both cell types are outlined in green in each panel. (**b**) Small region of an eye imaged live, with Myo-II in red and F-actin in green. At $t = 15$ s (left panel), the boundary between the 3° and 2° lattice cell is constricted (indicated with bracket) and Myo-II has accumulated. 17

at lattice cell: lattice cell junctions while Rho1 and Rok accumulation, which activate Myo-II (Heer and Martin 2017), correlated with junction contraction. While the precise role of pulsing at these lattice cell: lattice cell membranes is unclear, the authors suggest it allows 2° and 3° cells to sample different shapes on their pathway to acquiring the most favorable final shape.

The importance of actin and myosin in determining the final lattice cell size and shape is nicely demonstrated in retinas mosaic for myosin or actin activities. For example, when myosin activity is impaired in 3°s, their boundaries with neighboring 1° cells remain extended and the *myo-II* mutant 3° is large, to the detriment of neighboring 2°s that are smaller in size (Fig. 10c). Similarly, 2°s that lack myosin function retain extended boundaries with neighboring 3°s, distorting the shape of these 3°s (Fig. 10c). In contrast, when SCAR function is impaired in 3°s or 2°s, preventing Arp2/3-mediated actin network elaboration, the junctions between cells are reduced in length and the apical area of that mutant cells is small (Fig. 10d). In addition to the activities of actin and myosin networks, adhesion plays a key role in determining lattice cell shape and size. For example, ectopic expression of IRM proteins in single lattice cells enhances their adhesion to neighboring 1°s. This enables the lattice cell to expand its territory, at the expense of neighboring lattice cells (Fig. 10e) (Bao and Cagan 2005; Bao et al. 2010). Taken together, these data beautifully convey the impact that the formation and behavior of junctions and cytoskeletal structures in a lattice cell has on the size and shape of its connected neighbors. Indeed, that forces generated in one cell can be transmitted to its neighbors to influence their morphologies is apparent in a variety of tissues (Mao and Baum 2015) but how this phenomenon contributes to *Drosophila* eye patterning and how it is regulated has not been explored.

Regulation of Adhesion Receptors and the Cytoskeleton

When it comes to our understanding of how adhesion and the cytoskeleton are controlled, it is perhaps regulation of the IRM proteins that is best understood in the *Drosophila* pupal eye. The localization of IRM complexes at specific cell boundaries is crucial for correct eye patterning. These proteins are at first broadly expressed in the pupal eye but transcriptional regulation then limits them to specific cells. This coincides with recruitment of the 1° cells in which Notch signaling is activated (Cagan and Ready 1989b; Nagaraj and Banerjee 2007). Since *hbs* and *sns* transcription is activated downstream of Notch signaling, their expression is maintained in 1°s but lost from lattice cells which are occluded from *Delta*-expressing cone cells (Bao 2014; Krejci et al. 2009; Nagaraj and Banerjee 2007). Conversely, since Notch activity antagonizes *rst* and *kirre* expression, these are repressed in 1°s (Bao 2014). As a consequence, partnering IRM proteins are restricted to complementary expression domains and form adhesion complexes at boundaries between these. The IRM complexes are subsequently regulated by several mechanisms. Though poorly understood, these mechanisms include the adaptor protein Cindr that promotes IRM com-

plex accumulation at lattice cell: 1° cell junctions while also promoting IRM complex removal from lattice: lattice cell junctions (Johnson et al. 2012); the BAR domain protein PICK1 that is required for IRM complex stability (Hohne et al. 2011); and β-Spectrin, which binds Rst and the cell polarity determinant Crumbs, providing a mechanism to specifically localize IRM complexes to adherens junctions (Lee et al. 2010). Conversely, Pyd inhibits accumulation of IRM complexes at adherens junctions in the pupal eye (Seppa et al. 2008), although this is possibly a consequence of Pyd's modification of actomyosin structures to compromise adherens junctions, as has been shown in other systems (Choi et al. 2016; Fanning et al. 2012).

Actin, actomyosin structures, and adherens junctions are profoundly influenced by the activities of monomeric Rho family GTPases (Citi et al. 2014). Accordingly, the roles of Rho1, Cdc42, and Rac1 have been considered in the pupal eye and each is required for correct eye patterning. Specifically, since it promotes Myo-II activity, Rho1 has been shown to limit the apical areas of pupal eye cells (Warner and Longmore 2009b). Further, since Rho1 also promotes Formin activity, it is necessary for the correct structure of the cytoskeleton in pupal eye cells (Warner and Longmore 2009b). In addition, Rho1 has a crucial role in repressing the recycling endocytosis of E-Cad in the eye, and hence contributes to adherens junction stability (Warner and Longmore 2009b; Yashiro et al. 2014). Meanwhile, Cdc42 antagonizes Rho1 activity in the pupal eye (Warner and Longmore 2009a) and Rac1 has been shown to be crucial for the correct formation of adherens junctions (Bruinsma et al. 2007). However, in each of these studies, the focus was on the function of the GTPases *per se,* rather than their temporal and spatial contribution to specific events during eye morphogenesis and so it will be interesting to revisit how the GTPases function and are regulated with this perspective in mind.

It is likely that many mechanisms have evolved to regulate adherens junctions and the cytoskeleton in the eye and that redundancy between these mechanisms ensures robust and uniform eye patterning. These regulatory systems are likely to include transcriptional and post-translational regulation of junction and cytoskeletal proteins; the regulated assembly, distribution or clustering of adherens junctions; and the regulated activity and turnover of junction and cytoskeletal proteins (for reviews, see (Baum and Georgiou 2011; Bulgakova et al. 2012; Cavey and Lecuit 2009; Kowalczyk and Nanes 2012; Takeichi 2014; Troyanovsky 2012; Valenta et al. 2012). Of course, in the context of pupal eye patterning it is how these mechanisms are spatially and temporally regulated to affect morphogenesis that is most interesting. We eagerly await these analyses.

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Drosophila Eye as a Model to Study Regulation of Growth Control: The Discovery of Size Control Pathways

Shilpi Verghese, Indrayani Waghmare, Shree Ram Singh, and Madhuri Kango-Singh

Introduction

In the biological sense, the term growth has intricate ramifications that we have only started to comprehend. Growth is the overall increase in cell mass or size of a tissue or organism (Conlon and Raff 1999; Cook and Tyers 2007; Edgar 1999; Raff 1996). Growth may be due to increase in cell number resulting from cell division (cell proliferation), increase in cellular mass without cell division (cell enlargement), or release of more extracellular matrix (cell accretion). These processes are intimately linked, and it is clear that if coordinated growth has to occur in an organism, it is necessary for various biological pathways to interact and relay appropriate signals to proper cell types. Growth regulation is precisely controlled and affected by several intrinsic and extrinsic factors (Cooper 2004; Crickmore and Mann 2008;

Shilpi Verghese and Indrayani Waghmare contributed equally to this work.

S. Verghese
Department of Cell Biology, Emory University School of Medicine, Atlanta, GA, USA

I. Waghmare
Department of Cell and Developmental Biology, Vanderbilt University, Nashville, TN, USA

S. R. Singh
Mouse Cancer Genetics Program, National Cancer Institute, Frederick, MD, USA

M. Kango-Singh (✉)
Department of Biology, University of Dayton, Dayton, OH, USA

Center for Tissue Regeneration and Engineering at Dayton (TREND), University of Dayton, Dayton, OH, USA

Premedical Program, University of Dayton, Dayton, OH, USA

Integrative Science and Engineering Center (ISE), University of Dayton, Dayton, OH, USA
e-mail: mkangosingh1@udayton.edu

A. Singh, M. Kango-Singh (eds.), *Molecular Genetics of Axial Patterning, Growth and Disease in Drosophila Eye*,
https://doi.org/10.1007/978-3-030-42246-2_8

Grebien et al. 2005; Johnston and Gallant 2002). The intrinsic factors mainly involve synthesis and secretion of signals or ligands, which bind to their cognate receptors to relay downstream signals. These signals consist of a variety of molecules such as hormones, mitogens, apoptosis-inducing signals, patterning and axis-determining signals, etc. which eventually determine organ size and tissue homeostasis (Johnston and Gallant 2002; Mitchison et al. 1997; Montagne 2000; Tumaneng et al. 2012a). Growth of a tissue or organ is impacted not only by cell division but also by regulated cell death (apoptosis or programmed cell death) (Bangs and White 2000; Jacobson et al. 1997; Martin et al. 2009; Oldham et al. 2000a; Richardson and Kumar 2002; Rusconi et al. 2000).

In this chapter, we will focus on growth regulation in imaginal discs (epithelial sacs that are precursors of adult appendages) in *Drosophila melanogaster* (Bergantinos et al. 2010; Bryant 1978, 1987, 2001; Bryant and Schmidt 1990). The obvious advantages that *Drosophila* has to offer as a model organism include short life cycle, high fecundity, low-cost maintenance, and lack of redundancy in genome (Bier 2005; Blair 2003; Boutros and Ahringer 2008; Pagliarini et al. 2003; St Johnston 2002; Vidal and Cagan 2006). Furthermore, the sophisticated fly genetics provides great deal of versatility in terms of designing experiments. The plethora of knowledge thus generated through exhausting efforts of scientists has not only revealed to us classic information about how growth occurs but has also led to better understanding of growth-related diseases such as cancer.

Drosophila Eye as a Model to Study Regulation of Growth

The compound eyes of *Drosophila* arise from the eye-antennal imaginal discs, a monolayer epithelial sheet of cells that is responsible for the development of the eyes, the antennae, the ocelli, and a major part of the adult head cuticle. Each eye of the adult fruit fly on an average consists of about 800 ommatidia (Wolff and Ready 1993). Ommatidia arise from a set of 19 precursor cells that are generated by spatially and temporally coordinated cellular processes such as cell proliferation, cell differentiation, and cell death in the eye imaginal discs. Eighteen of these cells contribute to the eye per se, whereas the 19th cell gives rise to a sensory bristle (Cagan 1993). A key feature that distinguishes the eye from the rest of the organs is its ability to perceive light and relay the signal to distinct areas in the brain called the optic lobes. The eye imaginal discs arise from about 50 primordial cells that express the *Drosophila* PAX 6 gene eyeless (ey) during mid to late embryogenesis. Two such discs develop in each larva and differentiate into two compound eyes, antennae, ocelli, and the head cuticle in the adult.

Much is known about the regulation of growth and differentiation of the eye-antennal imaginal discs (Baker 2001; Cagan 1993; Dominguez and Casares 2005; Hafen 1991; Kramer and Cagan 1994; Kumar 2001). Until the second larval instar of development, the cells of the eye-antennal discs proliferate without differentiation (Baker 2001; Wolff and Ready 1993). During the second instar stage, a unique

process of cell differentiation begins in the eye-antennal disc that paves the way for formation of photoreceptor neurons in the posterior region of the eye-antennal imaginal disc (Wolff and Ready 1993). The differentiation occurs in the wake of a so-called morphogenetic furrow—a front marked by apical constriction of epithelial cells in response to complex developmental signaling from the Hedgehog, Dpp, Wg, and EGFR pathways (Acquisti et al. 2009; Chen and Chien 1999; Firth et al. 2010; Harvey et al. 2001; Kango-Singh et al. 2003; Penton et al. 1997). Posterior to the morphogenetic furrow, the cells begin to acquire particular photoreceptor cell fates and organize into ommatidial clusters.

Anterior to the furrow, cells divide asynchronously and do not differentiate; however, in the morphogenetic furrows, cells are arrested in the G1 phase of the cell cycle, synchronize, and either start to differentiate into photoreceptor cells as they leave the furrow or undergo one additional round of cell division, referred to as the second mitotic wave (SMW) before differentiating into the remaining photoreceptor, cone, pigment, and bristle cells (Baker 2001; Dickson and Hafen 1993; Wolff and Ready 1993). The cells posterior to the morphogenetic furrow enter G1 arrest caused by Dpp *(decapentaplegic)* signaling that is maintained by the *roughex (rux)* gene, which negatively regulates G1-S transition. The cells that are temporarily trapped in the G1 phase begin differentiation with specification of the R8 (photoreceptor) cell due to expression of the proneural protein Atonal (Ato) (Baker et al. 1996; Chen and Chien 1999; Daniel et al. 1999; Dominguez 1999; Greenwood and Struhl 1999; Jarman et al. 1994). R8 recruits other photoreceptor cells—R2, R3, R4, and R5—to form a cluster of five photoreceptor precursors. Once specified, these cells never enter cell cycle or cell division again. All other non-specified cells reenter cell cycle only once—a process referred to as the second mitotic wave (SMW) (Anon 2003; Baker 2001; de Nooij and Hariharan 1995). Cells in SMW undergo G2/M phase that is mediated through local signaling from Spitz (Spi). Binding of Spi to its cognate receptor EGFR in precursor cells causes activation of downstream *string (Bakal)* that completes the G2-M transition during mitosis. Local Spi-EGFR signaling also plays an important role limiting the progression of SMW. For instance, on an average, the Spi signal from one pre-cluster can span to a length of seven cells only causing these cells to divide, whereas the remaining cells remain arrested in G2 phase and fail to divide (Baker 2001; Brumby and Richardson 2003) (de Nooij and Hariharan 1995; Jarman et al. 1994; Price et al. 2002) (Wolff and Ready 1991). The progression of the morphogenetic furrow is complete by the mid-third instar of larval development, and the eye-antennal disc is fully grown to about 50,000 cells (Kumar 2009; Kumar and Moses 2000, 2001; Sun 2007).

Following development in larval stages, supernumerary cells are eliminated via apoptosis during pupal development. This event is mediated through Notch signaling (Bonini and Fortini 1999; Burke and Basler 1997; Sawamoto and Okano 1996; Treisman and Heberlein 1998; Zipursky 1989). By contrast, survival of pupal cells is brought about by EGFR expression that mediates its cell survival function through suppressing the transcriptional activity of the pro-apoptotic gene *head involution defective (hid)* (Bonini and Fortini 1999). In addition, survival signals emanating from cone or primary pigment cells in each ommatidium play a role in survival and

proliferation of secondary and tertiary pigment cells and secondary bristle organs (Cagan 1993, 2009; Rubin 1989; Singh et al. 2012; Tsachaki and Sprecher 2012; Yamamoto 1993). During metamorphosis, the two eye-antennal imaginal discs fuse at the dorsal midline to form the fly head with three ocelli, two antennae, and compound eyes. Thus, the eye-antennal disc is ideal for the study of organogenesis, morphogenesis, pattern formation, and several cell biological processes including the regulation of cell cycle, cell death, cell junctions and adhesion, transport of molecules, cell signaling, and metabolism. Recently, the eye discs have been used as an experimental system for genetic screens to discover postembryonic lethality and for screening small molecule inhibitors in chemical and drug screens.

The Mosaic Analysis Systems and the *Drosophila* Eye

Mutagenesis screen is a very well-established tool for gene discovery in flies [for review, see (Bellen et al. 2011, 1989; Blair 2003; Pfeiffer et al. 2010; St Johnston 2002; Venken and Bellen 2012; Xu and Rubin 1993)]. Over the years, the **mosaic techniques** have evolved to include the FLP-FRT, eyFlp, EGUF, eyFlp cl w+, Flp-out clones, and MARCM [for review, see (Blair 2003; St Johnston 2002)]. One of the first tissue-specific mosaic systems was developed in the eye-antennal discs where the mosaic clones were restricted to the eye-antennal discs by virtue of expression of the Flippase gene under the control of the eyeless promoter (commonly referred to as the "*ey-FLP* system") (Newsome et al. 2000). This tissue-specific system was further refined by the development of the "cell-lethal" system, where effects of loss of function of a gene could be surveyed more clearly because the wild-type twin clones are eliminated due to the presence of *cell-lethal* mutations (the *cell-lethal FLP-FRT* system) (Newsome et al. 2000). We focus on the genetic screens performed about 10–12 years ago (simultaneously in our labs) that lead to the identification of many new genes that were shown to belong to the two major growth regulatory networks: the Hippo pathway and the TSc-ToR pathway.

Genetic Screens for Genes That Regulate Growth: The "Big-Head" and "Pin-Head" Mutations

Barry Dickson's group (Newsome et al. 2000) improved the traditional FLP-FRT approach developed in the Rubin Lab (Xu and Rubin 1993), to allow generation of essentially mutant eye discs by eliminating the wild-type twin clone via a *cell-lethal* mutation (the *cell-lethal FLP-FRT* system) (Fig. 1). This so-called "cell-lethal" approach allows the mutant clones to grow to their highest potential due to elimination of competitive interactions between the mutant cells and their wild-type neigh-

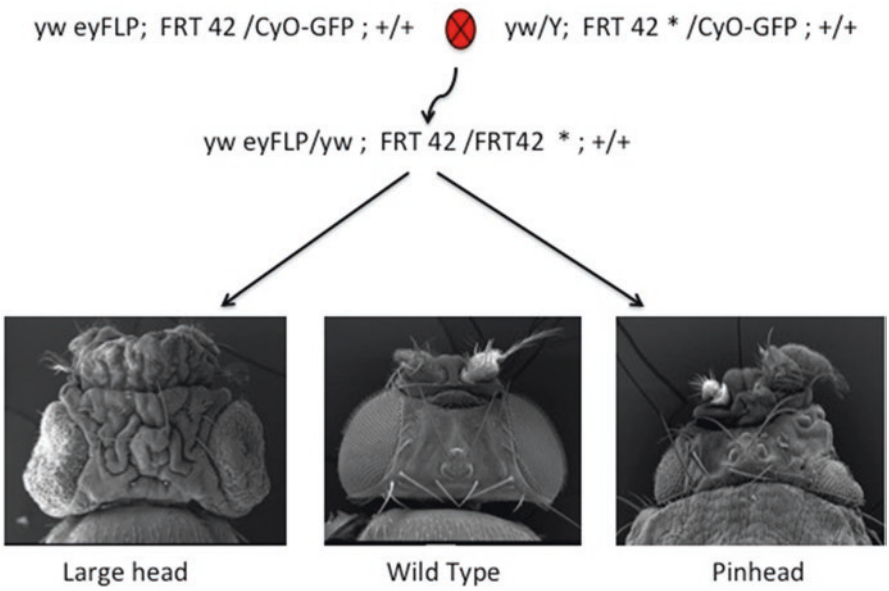

Fig. 1 Mutagenesis schemes for eye-specific mosaics lead to the identification of several Hippo and Tsc-TOR pathway mutants. (**a**) Modified mutagenesis scheme, (**b**) typical phenotypes of Hippo and Tsc-TOR pathway mutant from the mutagenesis screen

bors. Using this system, several groups carried out mutagenesis screens in flies (on the X, 2L, 2R, 3L, 3R chromosomes) and found mutations that affected patterning, growth, cell death, and differentiation [for review, see (St Johnston 2002)].

Of special interest were gene mutations which caused a remarkable effect on growth without disrupting the patterning process (Conlon and Raff 1999; Johnston and Gallant 2002; Mitchison et al. 1997; Oldham et al. 2000a; Raff 1996; Su and O'Farrell 1998; Tumaneng et al. 2012a). Characterization of these mutants revealed the mechanisms that regulate growth and tissue size by controlling cell number (Hippo pathway) (Zhao et al. 2011b) or cell size (InR/TSC-TOR pathway) (Kim and Guan 2011; Loewith 2011; Montagne 2000; Potter et al. 2003; Soulard et al. 2009) in a developing organ. Typically, loss-of-function mutations in positive regulators of these pathways caused development of enlarged heads that showed overgrowth—referred to as the "big-head" mutations (Hafen 2004; Oldham and Hafen 2003; Pan 2007, 2010). In contrast, loss-of-function mutations in negative regulators of these pathways caused reduction in head size and development of smaller organs, which may be due to cell death or reduction in cell size, and were referred to as the "pin-head" mutations.

The Hippo Signaling Pathway

The Hippo signaling pathway was first discovered in flies following characterization of "big-head" mutants identified from genetic screens [for review, see (Edgar 2006; Pan 2007; Saucedo and Edgar 2007)]. Analysis of the loss-of-function phenotypes revealed that a fundamental function of the Hippo pathway was the regulation of organ size (Boggiano and Fehon 2012; Harvey and Hariharan 2012; Schroeder and Halder 2012; Staley and Irvine 2012). Interestingly, the pathway received its name just after some growth regulatory genes [*warts* (*wts*), *salvador* (*sav*, aka *shar-pie*, *shrp*)] were characterized. Warts (wts) was named based on the bumpy "warts-like" phenotype of the mutant cells in mitotic (mosaic) clones on the body of the adult flies that were reminiscent of the warts on toads (Justice et al. 1995). Another group led by Xu et al. (1995) also independently found *warts* in the initial FLP/FRT-based screen and named it *large tumor suppressor (lats)* (Xu et al. 1995). Two independent groups identified the gene encoding the adaptor protein Salvador (Sav) (aka Shar-pie, Shrp after the dog species of the same name as the mutant flies showed a characteristic phenotype of folded dark cuticle on the overgrown heads) from complementation groups isolated from the big-head genetic screens (Kango-Singh et al. 2002; Tapon et al. 2002). Interestingly, both Wts and Sav regulated growth by suppressing proliferation and promoting apoptosis. Hippo was the name given to another complementation group from the "big-head" screens that showed a phenotype that was very similar to Wts and Sav (Harvey et al. 2003; Jia et al. 2003; Pantalacci et al. 2003; Udan et al. 2003; Wu et al. 2003).

Molecular analysis of the three genes revealed that Wts and Hpo genes encode for serine-threonine (S-T) kinases, whereas Sav is a WW domain-containing adaptor protein (Kango-Singh et al. 2002; Tapon et al. 2002). By this time, it was clear that Warts, Salvador, and Hippo all show similar loss-of-function phenotypes and control organ size by a common signaling pathway that promotes apoptosis and restricts cell proliferation (Edgar 2006; O'Neill and Kolch 2005; Rothenberg and Jan 2002), and the pathway got its name from the last member of this trio of genes. A complete pathway that relays a growth regulatory signal from the plasma membrane to the nucleus has emerged over the last decade. Although genetic mutagenesis screens led to the initial discovery of this pathway, several components were identified by other genetic screening strategies and biochemical approaches (e.g., yeast two-hybrid screens, TAP-TAG-based protein interaction assays) [for review, see (Halder and Johnson 2011; Kango-Singh and Singh 2009; Staley and Irvine 2012; Tumaneng et al. 2012a; Varelas and Wrana 2012)]. Today the Hippo pathway has grown to a large network of tumor suppressor genes that function upstream and downstream of the three initial members of the Hippo pathway (aka the core kinase cascade) that control several aspects of tissue homeostasis. Overall, the Hippo signaling pathway is a key size regulatory pathway that controls organ size in flies and vertebrates, and misregulation of Hippo signaling is implicated in several diseases including cancer [for review, see (Harvey and Hariharan 2012; Schroeder and Halder 2012; Staley and Irvine 2012; Zhao et al. 2011b)] (Fig. 2).

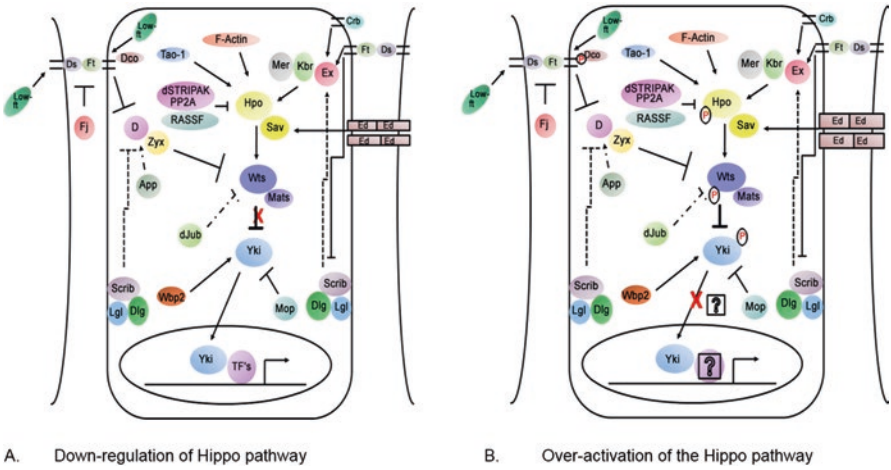

Fig. 2 Schematic representation of the Hippo pathway in *Drosophila melanogaster*. (**a**) Hippo pathway is downregulated in response to extracellular signals. Hippo (Hpo, #3206) fails to get phosphorylated and does not phosphorylate Warts (Wts). Inactive Wts cannot phosphorylate Yorkie (Yki) and allows Yki to enter the nucleus to bind cognate transcription factors and induce expression of target genes. (**b**) Hippo pathway is activated by stress, wherein Hippo (Hpo, #3206) is phosphorylated and in turn phosphorylates Warts (Wts) with the help of adaptor proteins Salvador (Sav) and Mats. Activated Wts phosphorylates Yorkie (Yki) and prevents it from entering the nucleus, thus preventing transcription of target genes. In addition, cell death is induced when the pathway is hyperactivated

Regulation by Core Kinase Cascade of the Hippo Pathway

The molecular analysis of the three initial members of the Hippo pathway in *Drosophila* revealed that Hpo codes for a S-T kinase of the mammalian sterile-20 family of kinases (Harvey et al. 2003; Jia et al. 2003; Pantalacci et al. 2003; Udan et al. 2003; Wu et al. 2003) and can physically associate with the WW domain-containing adaptor protein Sav (Harvey et al. 2003; Jia et al. 2003; Pantalacci et al. 2003; Udan et al. 2003; Wu et al. 2003). Wts is a S-T kinase protein of the DMPK family that associates with another adaptor protein Mob as tumor suppressor (Mats) (Justice et al. 1995; Lai et al. 2005; Shimizu et al. 2008; Wei et al. 2007; Xu et al. 1995). Loss of function of these genes in genetic mosaics revealed strong overgrowth phenotype caused by increased cell proliferation and diminished sensitivity to apoptosis. Hyperactivation of the pathway by overexpression of Hpo, Sav, Wts, or Mats leads to formation of smaller organs due to increased apoptosis (Harvey et al. 2003; Pantalacci et al. 2003; Udan et al. 2003; Wei et al. 2007; Wu et al. 2003). Biochemical analysis showed that the Hpo kinase phosphorylates and can physically associate with Sav, Wts, and Mats to form protein complexes in vitro (Wei et al. 2007) (Fig. 2). However, Hpo associates with its cognate adaptor protein Sav

to form the Hpo-Sav complex for efficient activation of the downstream kinase Wts (Huang et al. 2005; Wu et al. 2003). Wts itself associates with Mats to form the downstream Wts-Mats complex of the core kinase cascade of the Hippo pathway (Wei et al. 2007). Association of these adaptor proteins is known to stimulate the catalytic activity of the Hpo and Wts kinases (Dong et al. 2007; Pan 2007; Wei et al. 2007). Moreover, phosphorylation of Mats by the Hpo kinase increases its affinity for the Wts kinase (Dong et al. 2007; Pan 2007, 2010; Wei et al. 2007). Wts is activated by autophosphorylation and phosphorylation by Hpo kinase. Activated Wts associates with Mats (thus Mats cannot simultaneously associate with Hpo and Wts), which acts as a coactivator for the kinase activity of Wts (Dong et al. 2007; Huang et al. 2005; Oh and Irvine 2008, 2009). A major output of the core kinase cascade is to inhibit the growth-promoting activity of Yorkie (Yki), the *Drosophila* homolog of the mammalian YAP oncogene that acts as a transcriptional coactivator (Dong et al. 2007; Huang et al. 2005) (Fig. 2). Yorkie (Yki) was identified via a yeast two-hybrid screen as an interactor of Warts. Overexpression of Yki phenocopies the loss of function of *hpo*, *sav*, *wts*, and *mats* (all genes of the core kinase cascade) and causes overgrowth (Dong et al. 2007; Wei et al. 2007). Loss of function of *yki* results in formation of smaller organs due to induction of cell death (Huang et al. 2005).

Yki activity is regulated by controlling its subcellular localization via phosphorylation- dependent and phosphorylation-independent interactions with the core kinase cascade of the Hippo pathway (Oh and Irvine 2008, 2010; Ren et al. 2010b). Yki associates with Wts, and one mechanism by which the Wts kinase restricts Yki activity is via phosphorylation at Ser168 that creates a 14-3-3 protein-binding site (Goulev et al. 2008; Peng et al. 2009; Ren et al. 2010b; Wu et al. 2008; Zhang et al. 2008b, 2009a). Interestingly, only phosphorylated forms of Yki can associate with 14-3-3 proteins. Yki is phosphorylated at multiple sites (e.g., Ser 111 and S250), which increase Yki activity making it less sensitive to Hpo/Wts-mediated inhibition. These phosphorylation events act in parallel to phospho-Yki/14-3-3-mediated mechanisms and inhibit Yki nuclear localization and activity. It is suggested that nuclear export is required for shuttling Yki to the nucleus in response to Hpo signaling, and binding of 14-3-3 proteins is thought to impede nuclear import and/or promote nuclear export, thereby facilitating nucleocytoplasmic shuttling of target proteins (Brunet et al. 2002; Kumagai and Dunphy 1999). Nuclear transport of Yki depends on its binding with cognate transcription factors as Yki does not have an intrinsic nuclear localization signal (NLS) (Goulev et al. 2008; Zhang et al. 2008a, b) (Fig. 2). Currently, it is unclear if binding of 14-3-3 proteins to Yki prevents its binding with cognate transcription factors or masks the nuclear localization signals or promotes export from the nucleus. Nevertheless, coactivator Yki/YAP is the critical downstream regulatory target of the Hpo kinase cascade, and regulation of its subcellular localization is the primary mechanism by which the Hpo pathway influences target gene expression (Goulev et al. 2008; Huang et al. 2005; Oh and Irvine 2008, 2009, 2010; Oh et al. 2009; Peng et al. 2009; Ren et al. 2010b).

Yki (like Sav) is a WW domain-containing protein and interacts with the PPXY motifs in Wts (Huang et al. 2005; Kango-Singh et al. 2002; Tapon et al. 2002).

Besides Wts, the WW domains of Yki interact with the PPXY motifs present in other components of Hippo signaling pathway like Expanded (Ex), Hpo, WW domain-binding protein 2 (Wpb2), and Myopic (Gilbert et al. 2011) to regulate Hippo signaling via phosphorylation-independent mechanisms (Badouel et al. 2009; Gilbert et al. 2011; Oh et al. 2009; Zhang et al. 2011b). Another protein that acts via its WW domains is Kibra which associates with the PPXY motifs in Ex (and binds Mer in a WW domain-independent manner) (Baumgartner et al. 2010; Genevet et al. 2010). The identification of multiple proteins that act through the interaction between WW domains and PPXY motifs in the Hippo pathway suggests that these motif-specific interactions are important for regulation of Hippo signaling [reviewed in (Sudol 2010; Sudol and Harvey 2010)].

Yki Activity and Regulation of Expression of Target Genes

Hyperactivation of the pathway, for example, by overexpression of Hpo, leads to phosphorylation and activation of Hpo and Wts with the help of adaptor proteins Sav and Mats. Wts, in turn, phosphorylates the transcriptional coactivator Yki, which associates with 14-3-3 proteins and remains sequestered in the cytoplasm (Dong et al. 2007; Huang et al. 2005; Oh and Irvine 2008; Oh et al. 2009; Ren et al. 2010b). Analysis of adult and imaginal disc phenotypes reveals that overexpression of Hpo results in induction of ectopic apoptosis early in development in imaginal disc cells due to induction of caspase-dependent cell death (Hamaratoglu et al. 2006; Harvey et al. 2003; Udan et al. 2003; Verghese et al. 2012a). In mammalian cells, activation of MST1/2 and hyper-phosphorylation of YAP2 by MST2 and LATS1 kinase lead to activation of cell death. MST1/2 are known targets of caspases. Furthermore, YAP1/2 are known to interact with p73 via a PDZ domain in YAP and induce apoptotic target genes (Bertini et al. 2009; Sudol 2010; Sudol and Harvey 2010). However, these mechanisms of regulating apoptosis may not be conserved in flies because the site for caspase cleavage is not conserved in *Drosophila* Hpo (Wu et al. 2003), and *Drosophila* Yki does not have the conserved PDZ domain (Sudol and Harvey 2010). Nevertheless, Hpo overexpression in flies induces apoptosis through an alternate mechanism that does not involve caspase cleavage or p73. Recently, it was shown that the effector caspase Dronc (*Drosophila* homolog of mammalian caspase-9) is induced in conditions when Hippo pathway is hyperactivated. Further, using reporter genes, it was shown that dronc transcription is induced during gain-of-function and downregulated during loss-of-function conditions of the Hippo pathway, suggesting that *dronc* is a transcriptional target of the Hippo pathway (Verghese et al. 2012a). However, the molecular mechanism by which Yki interacts with Dronc remains unclear. Both phosphorylation-dependent (e.g., with 14-3-3 by phosphorylation-dependent mechanisms) and phosphorylation-independent mechanisms (binding with Hpo, Wts, or Ex) result in cytoplasmic retention of Yki in multiple protein complexes. Thus, the possibility remains that hyperactivation of Hippo pathway releases Yki from one or more cytoplasmic com-

plexes to allow its binding to transcription factors and shuttle into the nucleus to induce *dronc* transcription. Alternatively, hyperactivation of the Hippo pathway involves a transcriptional repressor that acts together with or independent of Yki to control *dronc* expression. Thus, although it is clear that hyperactivation of the Hippo pathway leads to induction of apoptosis, the molecular mechanisms underlying this process are yet unidentified.

When the pathway is downregulated, the genes of the core kinase cascade act as tumor suppressors by suppressing the growth-promoting activity of Yki (Fig. 2). Under these conditions, Yorkie can partner with transcription factors like the TEAD family protein, Scalloped (Sd), and enter the nucleus and cause transcription of target genes which regulate cell proliferation and apoptosis. Sd was identified as the transcriptional factor of the pathway via yeast two-hybrid screen and in vitro Yki activity assays (luciferase assay) (Goulev et al. 2008; Wu et al. 2008; Zhang et al. 2008b). Sd is required for wing development (Campbell et al. 1992; Liu et al. 2000), whereas Yki is required for regulating growth of all imaginal disc cells. Other transcription factors that bind Yki to regulate growth via Hippo signaling have since been discovered. These include Mothers Against Dpp (Mad) (Alarcon et al. 2009; Oh and Irvine 2010; Peng et al. 2009), Homothorax (Hth), and Teashirt (Tsh) (Peng et al. 2009). Mad is a known transcription factor within the Dpp/TGFβ signaling pathway, and Mad and Hth were shown to control the activity of the *bantam miRNA* (Alarcon et al. 2009; Peng et al. 2009). Mad, Hth, and Tsh are known transcription factors that respond to other signals and are required for patterning of imaginal discs during development.

Yki activity is controlled by the upstream signals (Grusche et al. 2010; Oh and Irvine 2010) (Fig. 2). A large number of target genes have been identified over the past decade, which include the cell cycle regulators E2F1 and *cyclins E*, *A, B, and D*; the growth promoter *Myc* and cell survival-promoting miRNA *bantam*; genes regulating cell death like the *Drosophila inhibitor of apoptosis diap1*, *hid*, *and dronc*; and cytoskeletal proteins like F-actin, which drive cell proliferation and cell survival (Fig. 3) (Goulev et al. 2008; Harvey et al. 2003; Huang et al. 2005; Jia et al. 2003; Kango-Singh et al. 2002; Neto-Silva et al. 2010; Nolo et al. 2006; Pantalacci et al. 2003; Peng et al. 2009; Tapon et al. 2002; Thompson and Cohen 2006; Udan et al. 2003; Wu et al. 2003, 2008; Zhang et al. 2008a; Ziosi et al. 2010). Yki also controls the expression of several upstream components of the Hpo pathway like Ex, Mer, Kibra, Crumbs (Crb) and Four-jointed (Fjose et al. 1984) by a negative feedback loop (Cho et al. 2006; Fjose et al. 1984; Genevet et al. 2009, 2010; Hamaratoglu et al. 2006). Recently, Yki was shown to affect the expression of components of other signaling pathways, such as ligands for the Notch, Wnt, EGFR, and Jak-Stat pathways (Cho et al. 2006; Karpowicz et al. 2010; Ren et al. 2010a; Shaw et al. 2010; Staley and Irvine 2010, 2012; Zhang et al. 2009a). These interactions suggest that Hippo pathway interacts with the major signal transduction pathways, and these points of contact between different pathways may play an important role in controlling correct tissue sizes and maintaining homeostasis (Fig. 3).

Genetic and biochemical studies thus provide a basic premise for how Yki activity is modulated when Hippo signaling is down- or upregulated (Halder and Johnson

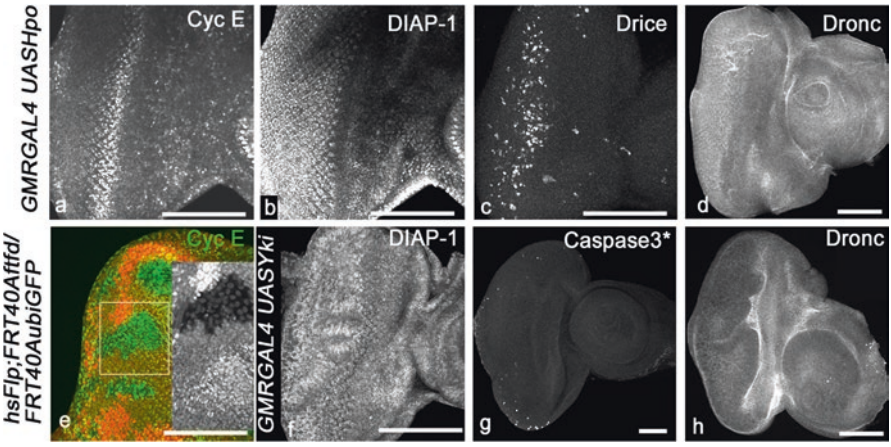

Fig. 3 Hippo pathway target genes regulate cell proliferation and apoptosis: (**a**–**d**, #6887) *GMRGAL4 UASHpo* third instar eye-antennal imaginal disc showing effect on target proteins upon pathway hyperactivation in the GMR domain. (**a**) Cyc E is downregulated, (**b**) DIAP-1 levels remain unaffected, and (**c**) Drice is activated (Drice is the homolog of *Drosophila* Caspase3∗ and is a readout of active Dronc). (**d**) Dronc is upregulated in the GMR domain upon Hpo overexpression. (**e**) Loss-of-function clones of *ft* (GFP negative) made with yw hsFLP; UbiGFP [*hsFLP; FRT40A* ft^{fd}*/FRT40A ubiGFP*] show upregulation of Cyc E in the mutant cells. This effect is very strong in the region of the second mitotic wave (SMW). (**f**–**h**) *GMRGAL4 UASYki* third instar eye-antennal imaginal discs. (**f**) DIAP-1 is upregulated, (**g**) Caspase3∗ staining is not observed, and (**h**) Dronc is downregulated in the GMR domain consistent with overproliferation and no apoptosis

2011; Harvey and Hariharan 2012; Schroeder and Halder 2012; Staley and Irvine 2012). Studies in imaginal discs and other cell types like intestinal stem cells and fat cells revealed that Hippo signaling is needed in all cell types to regulate growth and that the activity of the pathway is modulated to achieve tissue homeostasis (Halder et al. 2012; Halder and Johnson 2011; Harvey and Hariharan 2012; Tumaneng et al. 2012a; Zhao et al. 2008a, 2010a). Whether Hippo signaling pathway is regulated by other global instructive signals (e.g., morphogen gradients) or if the pathway is constitutively active remains unknown. However, several inputs that communicate a growth regulatory signal to the core kinase cascade have been identified. We will discuss the key inputs and their connection to the core kinase cascade in the following sections.

Upstream Regulators of the Hippo Pathway

Since the discovery of the core kinase cascade, several upstream regulators of the Hippo pathway were identified (Table 1). These discoveries highlighted two remarkable properties of the Hippo pathway—one, that the Hippo pathway is a signaling network with multiple points of signal integration rather than a linear system of

Table 1 Hippo pathway components and their biological roles

	Gene name, *symbol* [Chr]	Nature of protein	Role	References
Upstream regulators	Crumbs *Crb* [3]	Protein kinase C binding	Organization of adherens junction, establishment of cell polarity, photoreceptor and rhabdomere development	Fan et al. (2003), Pichaud and Desplan (2001), Tepass et al. (1990)
	Expanded *ex* [2]	Protein binding	Compound eye, photoreceptor cell differentiation, negative regulation of Hippo signaling cascade	Maitra et al. (2006), Pellock et al. (2007), Badouel et al. (2009), McCartney et al. (2000)
	Merlin *Mer* [1]	Protein binding	Regulation of programmed cell death, negative regulator of Hippo signaling	Pellock et al. (2007), Hamaratoglu et al. (2006)
	Kibra *Kibra* [3]	Protein binding	Compound eye morphogenesis, regulation of Hippo signaling cascade	Ling et al. (2010), Genevet et al. (2010), Yu et al. (2010), Baumgartner et al. (2010)
Fat branch	Fat *ft* [2]	Cell adhesion molecule binding	Establishment of planar polarity, negative regulation of growth, imaginal disc growth	Yang et al. (2002), Mao et al. (2006), Torok et al. (1993), Garoia et al. (2000), Matakatsu and Blair (2006)
	Low fat lft [2	Protein binding	Wing morphogenesis	Mao et al. (2009)
	Dachs D [2]	ATPase activity (predicted nature)	Establishment of ommatidial planar polarity, positive regulation of growth	Mao et al. (2006)
	Dachsous Ds [2]	Cell adhesion molecule binding	Eye morphogenesis, establishment of cell polarity, cell proliferation	Baena-Lopez et al. (2005), Clark et al. (1995)
	Four-jointed Fj [2]	Wnt-protein binding; protein kinase activity	Imaginal disc growth, establishment of planar polarity	Villano and Katz (1995), Bosveld et al. (2012)
	Scribbled Scrib [2]	Protein binding	Establishment of ommatidial planar polarity, negative regulation of imaginal disc growth	Courbard et al. (2009), Zeitler et al. (2004), Verghese et al. (2012)
	Zyxin Zyx [4]	Protein binding	Positive regulation of imaginal disc growth	Rauskolb et al. (2011)
	Approximated App [3]	Protein-cysteine S-palmitoyltransferase activity (predicted nature)	Establishment of body hair or bristle planar orientation	Matakatsu and Blair (2008)
	Discs overgrown Dco [3]	Kinase activity	Establishment of ommatidial planar polarity, positive regulation of cell growth	Strutt et al. (2006), Klein et al. (2006), Guan et al. (2007)

Core kinase cascade	Warts Wts [3]	Protein binding, kinase activity	Negative regulation of cell proliferation, R8 cell fate specification	Justice et al. (1995), Mikeladze-Dvali et al. (2005)
	Mob as tumor suppressor Mats [3]	Protein binding	Cell proliferation	Lai et al. (2005)
	Hippo Hpo [2]	Protein binding; serine/threonine kinase activity	Negative regulation of cell proliferation, R8 cell fate specification	Udan et al. (2003), Mikeladze-Dvali et al. (2005)
	Salvador Sav [3]	Protein binding	Negative regulation of cell proliferation, R8 cell fate specification	Kango-Singh et al. (2002), Mikeladze-Dvali et al. (2005)
Other regulators	Ajuba Jub [1]	Ligand-dependent nuclear receptor binding	Positive regulation of organ growth	Das Thakur et al. (2010)
	Tao Tao [1]	Serine/threonine kinase activity	Negative regulation of organ growth	Poon et al. (2011)
	Echinoid Ed [2]	Protein binding	Negative regulation of Hippo signaling cascade	Yue et al. (2012)
	Pez Pez [2]	Protein tyrosine phosphatase activity	Negative regulation of Hippo signaling cascade	Poernbacher et al. (2012)
	d-STRIPAK PP2A Pp2A-29B [2]	Serine/threonine phosphatase activity	Centrosome organization	Dobbelaere et al. (2008)
	Ras association family member Rassf [3]	Protein binding	Negative regulation of signal transduction	Polesello et al. (2006)
	Par-6 Par-6 [1]	Protein binding	Cell adhesion	Kiger et al. (2003)
	Atypical protein kinase C a-PKC [2]	Protein binding; serine/threonine kinase activity	Compound eye retinal cell programmed cell death, establishment of epithelial cell planar polarity	Ogawa et al. (2009), Kaplan et al. (2011)
	Stardust Sdt [1]	Protein binding	Zonula adherens assembly	Nam and Choi (2003), Bachmann et al. (2001)

(continued)

Table 1 (continued)

	Gene name, *symbol* [Chr]	Nature of protein	Role	References
	Lethal 2 giant larvae L2gl [2]	Myosin II binding; myosin binding	Cell competition in a multicellular organism, establishment of epithelial cell planar polarity	Tamori et al. (2010), Kaplan and Tolwinski (2010)
	Myopic Mop [3]	Protein tyrosine phosphatase activity	Regulation of growth	Gilbert et al. (2011)
	Patj dPatj [3]	Protein kinase C binding	Adherens junction organization	Nam and Choi (2006)
Transcription factors/ coactivators	Yorkie Yki [2]	Protein binding; transcription coactivator activity	Cell competition in a multicellular organism, cell proliferation	Ziosi et al. (2010), Huang et al. (2005), Thompson and Cohen (2006)
	Scalloped Sd [1]	Transcription factor binding	Compound eye morphogenesis	Garg et al. (2007)
	Homothorax Hth [3]	Protein binding; transcription factor	Compound eye photoreceptor fate determination	Wernet et al. (2003)
	Teashirt Tsh [2]	Transcription factor activity	Eye-antennal disc development	Singh et al. (2004)
	Wpb2 Wbp2 [3]	Transcription factor binding	Positive regulation of imaginal disc growth	Zhang et al. (2011a, b)
	Mothers against dpp Mad [2]	Transcription factor activity	Compound eye morphogenesis, negative regulation of gene expression	Cordero et al. (2007), Anderson et al. (2006)

epistatic genes (Fig. 2), and two, the interactions between various protein complexes (at the signal integration points) may play a decisive role in shaping the outcome, i.e., Yki activity levels. Although our understanding of the network is incomplete in both these areas, it is clear that signaling interactions within this pathway are shaped by several distinct inputs.

Fat Signaling and the Hippo Pathway

fat (ft) alleles were spontaneous mutations first described by Mohr (1923, 1929). Subsequent analysis of mutations in the *ft* locus revealed both viable and lethal alleles, of which the null alleles are larval lethal and show hyperplastic overgrowth of imaginal discs thereby acting as tumor suppressor genes (Bryant et al. 1988). Molecular cloning of *ft* revealed that it codes for a transmembrane protein, which is an atypical cadherin (Mahoney et al. 1991). Loss of *ft* affects two distinct aspects of imaginal disc growth and development, restriction of cell proliferation and generation of correctly oriented cells within the epithelial sheet, phenotypes that were mapped to two distinct signaling pathways—the Hippo and the planar cell polarity (PCP) pathway (see (Cho 2006 #659) (Brittle et al. 2010; Matakatsu and Blair 2006, 2008, 2012)]. Ft is ubiquitously expressed; however, its functions are regulated by two genes, Dachsous (Ds) and Fj, which are expressed in gradients in developing tissues (Matakatsu and Blair 2004; Reddy and Irvine 2008). Ds is another protocadherin in flies that acts as the ligand for Ft for both the Hippo and PCP pathways [reviewed in (Thomas and Strutt 2012)]. Fj is a Golgi-localized kinase that phosphorylates the extracellular cadherin domains of Ft and Ds to promote their binding (Ishikawa et al. 2008; Simon et al. 2010). Phosphorylation of Fat by Fj increases its affinity to Ds, while phosphorylation of Ds reduces its affinity to Ft. One way in which Fat regulates growth and PCP is based on the slope and vector of the Ds and Fj gradients (Halder and Johnson 2011; Willecke et al. 2008; Zecca and Struhl 2010) (Fig. 2).

Several years after Ft was discovered, it was realized that the growth regulatory functions of Fat were tied to the Hippo pathway (Bennett and Harvey 2006; Cho et al. 2006; Silva et al. 2006; Willecke et al. 2006). Loss of *ft* in mutant clones phenocopied the loss-of-function phenotypes of genes within the core kinase cascade of the Hippo pathway. Imaginal discs containing somatic clones of *ft* mutant cells continued to proliferate when normal cells had stopped, thereby forming large overgrown discs. Transcriptional targets of Hippo pathway are induced within the *ft* mutant cells, a phenotype similar to loss of function of positive regulators of Hippo pathway (*e.g., wts, Hpo, sav, mats*). Ft affects the levels and localization of Hippo pathway components, including Wts, Ex, and Yki (Bennett and Harvey 2006; Cho et al. 2006; Oh and Irvine 2008; Silva et al. 2006; Tyler and Baker 2007; Willecke et al. 2006). Ft influences Hippo signaling independent of other upstream regulators like *expanded*, *merlin (mer)*, *and kibra* which form a heteromeric complex (Ex-Mer-Kibra) and other genes like the Tao-1 kinase (Boggiano et al. 2011; Poon et al.

2011) that act upstream of Hpo (Boggiano and Fehon 2012). However, several other genes were recently identified that specifically act downstream of Ft and integrate with the Hippo pathway by influencing the activity of the downstream kinase Wts. Thus, the Fat branch of the Hippo pathway has emerged that independently influences Wts activity and tissue growth (Halder and Johnson 2011; Kango-Singh and Singh 2009; Reddy and Irvine 2008; Staley and Irvine 2012) (Fig. 2).

Several components of the Ft branch influence the intracellular domain of Ft—the region critical for transducing the signal within cells (Fig. 2). These include the *Drosophila* Discs overgrown (Dco, #6929), a homolog of casein kinase I, which phosphorylates the Ft intracellular cytoplasmic domain in a Ds-dependent manner (Cho et al. 2006; Feng and Irvine 2009; Sopko et al. 2009), and the unconventional myosin Dachs (D) (Cho et al. 2006; Cho and Irvine 2004; Mao et al. 2006). Loss of function of dco^{3}, a hypomorphic allele, in homozygous discs and in somatic clones results in tissue overgrowth and shows elevated levels of Fj and Diap-1 (Bryant and Schmidt 1990; Feng and Irvine 2009; Guan et al. 2007). Dco binds to the cytoplasmic domain of Fat, and in *dco* mutants, Fat intracellular domains fail to phosphorylate. Ds enriches availability of Fat at the point of cell contacts by forming *cis*-dimers with Fat. This promotes the transphosphorylation of Fat by Dco. Lowfat is a novel protein that interacts with the intracellular domains of Fat and Ds and stabilizes the Fat-Ds interaction (Mao et al.). Lowfat was identified in a genome-wide yeast two-hybrid screen as a Fat- and Ds-interacting protein (Mao et al. 2006, 2009). In addition, the palmitoyltransferase Approximated (App) acts downstream of Ft, and Ft regulates the localization of D to the membrane through APP (Matakatsu and Blair 2008). Recently, the apical-basal polarity gene *scribble (scrib)* (Verghese et al. 2012b) and the LIM-domain protein *zyxin 102* (*zyx*) (Rauskolb et al. 2011) were shown to act in the Fat branch of Hippo signaling pathway (Bennett and Harvey 2006; Cho et al. 2006; Meignin et al. 2007; Polesello and Tapon 2007; Reddy et al. 2010; Silva et al. 2006; Willecke et al. 2006).

The differences in Ds and Fj expression between neighboring cells stimulate Yki activity, whereas the vector property of the gradients affects PCP signaling. Localization of D to the membrane is regulated by Fj, Ds, and Ft (Cho et al. 2006; Mao et al. 2006; Rogulja et al. 2008; Willecke et al. 2008). D controls Yki activity by two alternative mechanisms: the first involves posttranslational effects of Ft on Wts, and the second involves the localization of Ex to the subapical membrane (Bennett and Harvey 2006). The apical-basal polarity gene *scrib* and the atypical myosin D are responsible for partitioning the growth regulatory signal from Ft to downstream genes. Genetic epistasis experiments placed Ft upstream of D and the apical regulator of the pathway—Expanded (Ex) (Cho et al. 2006; Mao et al. 2006; Verghese et al. 2012b). D can reverse the effects of loss of *ft* on growth and expression of Fat target genes like Wg, Serrate, and Fj (Mao et al. 2006). Scrib was also placed upstream of D and Ex and downstream of Ft based on genetic epistasis experiments (Verghese et al. 2012b) (Fig. 2). When Ft is inactive, D is regulated by Approximated (App) (Matakatsu and Blair 2008). App posttranscriptionally modifies D and affects its localization at the apical cell cortex. Hence, App functions in the Hippo pathway by affecting the availability of D at the apical cell cortex. When

Ft is activated, D is released from App and binds to Zyxin (Zyx), which in turn interacts with Wts and stabilizes Wts activity (Rauskolb et al. 2011). Zyx binds to D; genetic epistasis experiments placed Zyx downstream of Ft and Dco and upstream of Wts (Feng and Irvine 2007, 2009; Rauskolb et al. 2011). Thus, influencing Wts stability is a primary mechanism by which Ft controls growth via Hippo signaling (Fig. 2). However, the other input via Ex remains less clear although there is clearly an input from Ft to Ex that also contributes to the Fat-branch-related phenotypes and regulation of the Hippo signaling pathway. Whether Fat signaling simultaneously signals through Ex (and the core kinase cascade) and D or the signals downstream of Ft are partitioned to allow maximum and more efficient signal transduction to the core kinase cascade remains unknown. Currently, the possibility that certain extracellular signals preferentially transmit the signal to Ex or D downstream of Ft has not been addressed.

Apical Membrane Proteins of the Hippo Pathway

Over the last 5 years, it has become clear that membrane-localized proteins are an intrinsic part of the Hippo signaling pathway (Genevet and Tapon 2011; Grusche et al. 2011; Halder et al. 2012; Schroeder and Halder 2012) (Table 1). Among these are the cell polarity proteins and proteins required for maintaining the cytoskeleton (Fig. 2). The FERM domain-containing adaptor proteins Ex and Merlin (Mer) were among the earliest Hippo pathway components that were known to localize to the apical membrane (Hamaratoglu et al. 2006; McCartney et al. 2000). Ex and Mer act upstream of the Hpo kinase and regulate pathway activation (Hamaratoglu et al. 2006). Loss of *mer* and *ex* together in somatic clones caused dramatic overproliferation of cells leading to overgrowths. These effects were synergistic because loss of function of *ex* or *mer* alone does not cause similar defects. These genes function together to control proliferation by regulating expression of transcriptional targets of Hippo pathway (e.g., cyclin E and DIAP1). Expanded can also regulate the pathway by independently interacting with Yki and sequestering it in the cytoplasm (Badouel et al. 2009; Oh et al. 2009).

Another protein that binds Ex and Mer and acts upstream of Hpo is the WW and C2 domain-containing adaptor protein Kibra. Ex, Mer, and Kibra form a complex at the apical membrane in epithelial cells, which then activates the downstream core kinase cascade (Baumgartner et al. 2010; Cho et al. 2006; Genevet et al. 2010; Hamaratoglu et al. 2006; Pellock et al. 2007; Tyler and Baker 2007) (Fig. 2). Kibra was identified via a genome-wide screen in *Drosophila* and in S2 cells for candidates that modified Yki activity (Baumgartner et al. 2010; Genevet et al. 2010; Yu et al. 2010). Genetic epistasis experiments placed Kibra upstream of Hpo and Yorkie. Kibra affects the phosphorylation of Hpo and Yorkie. Kibra acts synergistically with Ex and Mer to regulate Wts phosphorylation, and Kibra binds to Sav and Hpo in a Sav-dependent manner (Baumgartner et al. 2010; Genevet et al. 2010).

Cell polarity genes have been well characterized in flies and mammalian model systems, and recent studies reveal a role for cell polarity genes in the regulation of Hippo signaling (Table 1, Fig. 2) (Genevet and Tapon 2011; Grusche et al. 2010; Grzeschik et al. 2007, 2010a; b; Schroeder and Halder 2012). Crumbs (Crb), a transmembrane protein, is the upstream regulator that regulates Ex activity (Chen et al. 2010; Ling et al. 2010; Robinson et al. 2010). Crb is required for proper localization of Ex. Crb regulates Yki activity by interacting with Expanded (Chen et al. 2010; Grzeschik et al. 2010a; Robinson et al. 2010). Crb was found through a genetic screen, and loss and gain of function of Crb cause overgrowth of tissues and upregulation of the Hippo pathway target genes. Echinoid (Ed) is another upstream regulator of the Hippo pathway that like kibra interacts with both Ex and Yki (Baumgartner et al. 2010; Genevet et al. 2010; Yu et al. 2010; Yue et al. 2012). Cells mutant for *ed* cause mislocalization of Sav from the subapical membrane without affecting Ex or Mer localization. Ed also interacts physically with Hpo, Ex, Mer, and Kibra (Yue et al. 2012).

F-actin acts as an upstream regulator of the Hippo pathway (Fig. 2). Increased levels of F-actin inhibit the pathway, and activation of Hippo pathway inhibits F-actin accumulation (Fernandez et al. 2011; Richardson 2011; Sansores-Garcia et al. 2011). Tao-1 phosphorylates Hpo and acts upstream of Hpo at T195 (Boggiano and Fehon 2012; Boggiano et al. 2011; Poon et al. 2011). RNAi knockdown of Kibra, Ex, and Mer (KEM) resulted in a significant decrease of endogenous Hpo protein in the membrane fraction (Boggiano and Fehon 2012; Boggiano et al. 2011; Poon et al. 2011). Thus, the apical proteins regulate Hpo at least in part by bringing the latter to the membrane, where Hpo may be activated via mechanisms yet to be determined.

Negative Regulators of the Hippo Pathway

Several members of the Hippo pathway were identified based on their effects on tissue growth, and the loss-of-function phenotypes of these components showed dramatic outgrowths and benign lesions in fly epithelia (Table 1). It was clear that additional components that keep this pathway in check (e.g., phosphatases or kinase inhibitors) must exist, as Hippo activity would need to be modulated both positively and negatively for maintaining tissue homeostasis. Thus, the search for negative regulators began that yielded many important and critical regulators of the Hippo pathway. Among the first genes identified in this category was the Ras Association Family (RASSF) gene, *dRASSF1* (Polesello et al. 2006) (Fig. 2). The dRASSF protein negatively regulates the pathway by inhibiting the phosphorylation of Hpo, thus interrupting the Hpo kinase from signaling to the downstream kinase Wts (Polesello et al. 2006; Scheel and Hofmann 2003). Other inhibitors that act by dephosphorylating Hpo are the phosphatases—striatin-interacting phosphatase (STRIPAK) and protein phosphatase 2A (PP2A) (Ribeiro et al. 2010) (Fig. 2). A second mechanism of inhibition of Yki activity was identified by the *Drosophila* Ajuba family gene,

djub (Das Thakur et al. 2010) (Fig. 2). Loss of *djub* in mutant clones in imaginal discs caused reduced proliferation and increased apoptosis, akin to *yki* mutant clones. Genetic interaction studies showed that *djub* acts downstream of Hpo but upstream of Yki and Wts (Das Thakur et al. 2010). Furthermore, Djub can physically associate with Wts and Sav and influence the signaling activity of Yki. Thus, *djub* negatively regulates the Hippo signaling by interfering with Yki phosphorylation and its subcellular localization (Das Thakur et al. 2010). Recently, another negative regulator, *myopic* (Bonner and Boulianne 2011), was identified in a genetic screen for conditional growth suppressors (Gilbert et al. 2011) (Fig. 2). *mop* encodes the *Drosophila* homolog of human *His domain protein tyrosine phosphatase* gene (*HD-PTP* or *PTPN23*) (Toyooka et al. 2000). *mop* mutant cells show overgrowth phenotypes due to a block in cell death. This growth is accompanied by upregulation of a subset of Yki transcriptional targets but not the antiapoptotic gene *diap1*. *mop* interacts genetically with *yki* and acts downstream of *wts* but at the level of *ex* and *yki*. Myopic PPxY motifs bind conserved residues in the WW domains of the transcriptional coactivator Yorkie, and Myopic colocalizes with Yorkie at endosomes (Gilbert et al. 2011). Thus, several negative regulators of the Hippo pathway are now known; however, much remains unknown about their mechanism of action and their influence on growth regulation during development (Tables 1 and 2).

Hippo Pathway Cross-Talks with Other Pathways

Hippo pathway is known to interact with other pathways to regulate growth (Table 2). In mice it has been shown that Mst2 interacts with Raf-1 of the ERK/MAPK pathway (Graves et al. 1998). Raf-1 inhibits dimerization of Mst2 and recruits a phosphatase to dephosphorylate Mst2, thereby inactivating it, a function independent of

Table 2 Pathways known to interact with the Hippo network

Pathway interactions	Responses	References
JNK pathway	Cell competition, compensatory proliferation, regeneration, cytoskeletal integrity, tumorigenesis	Chen et al. (2012), Sun et al. (2011), Densham et al. (2009), Enomoto et al. (2012)
Wingless pathway	Growth control	Verelas et al. (2010)
EGFR pathway	Growth control	Herranz et al. (2012)
Decapentaplegic pathway	Growth control	Rogulja et al. (2008)
Hedgehog pathway	Growth control, neuronal differentiation	Kagey et al. (2012), Lin et al. (2012)
Notch pathway	Neural stem cell maintenance, polar cell fate during oogenesis, cell differentiation, proliferation	Li et al. (2012), Chen et al. (2011), Yu et al. (2008)
TSC-TOR pathway		Latest paper from Tapon

the MAPK pathway (O'Neill and Kolch 2005). More recently, many points of intersection between Hippo and other signaling pathways have come to light. For example, in the last 5 years, Hippo pathway was shown to interact with JNK pathway to regulate compensatory proliferation, regeneration, and tumor progression (Chen et al. 2012; Doggett et al. 2011; Grzeschik et al. 2010a; Staley and Irvine 2010; Sun and Irvine 2010, 2011; Tyler et al. 2007; Varelas et al. 2010a). Furthermore, Hippo pathway interacts with Wingless/Wnt pathways in flies and mammals (Varelas et al. 2010a, b). Hippo pathway restricts Wnt/beta-catenin signaling by promoting an interaction between TAZ and DVL in the cytoplasm. TAZ inhibits the CK1delta/epsilon-mediated phosphorylation of DVL, thereby inhibiting Wnt/beta-catenin signaling (Azzolin et al. 2012; Tsai et al. 2012; Varelas et al. 2010a). In *Drosophila*, Hippo signaling modulates Wg target gene expression (Varelas et al. 2010a, b). More connections of Hippo signaling with pathways that control morphogenetic patterning and growth have been uncovered which include the discovery of the regulation of TGF beta/SMAD complexes by YAP/TAZ in mammalian models and Yki in flies (Chan et al. 2011; Meignin et al. 2007; Polesello and Tapon 2007; Rogulja et al. 2008; Sudol and Harvey 2010; Varelas et al. 2010b). Dpp (Decapentaplegic) signaling interacts with D to maintain Fj and Ds gradient in order to regulate proliferation in the wing (Rogulja et al. 2008). Hippo pathway also intersects the PI3K/TOR pathway via multiple interactions (Bellosta and Gallant 2010; Collak et al. 2012; Karni et al. 2008; Kim et al. 2010; Mills et al. 2008; Sekido 2008; Strassburger et al. 2012; Tumaneng et al. 2012a, b; Wehr et al. 2013), with G-protein-coupled receptor (GPCR) signaling (Yu et al. 2012) and receptor tyrosine kinase signaling (Gadd et al. 2012; Garami et al. 2003). In fact, the web of interactions has grown exponentially over the last few years such that oftentimes the Hippo pathway is sometimes referred to as a network or superhighway (Barry and Camargo 2013) (Fig. 4).

Mammalian Hippo Pathway

Hippo pathway is responsible for regulating organ size and is involved in regeneration (Bertini et al. 2009; Hiemer and Varelas 2013; Hong and Guan 2012; Liu et al. 2012a). The core kinase pathway is highly conserved in mammals (Hong and Guan 2012; Liu et al. 2012a; Zhao et al. 2008a). In vertebrate models, the core kinase cascade consists of Mst1/2 (Hpo homolog) and Lats1/2 (Wts homolog) along with their adaptor proteins WW45 (Sav) and MOB1 (Mats homolog), which control growth by regulating phosphorylation of YAP (Yki homolog) (Hong and Guan 2012; Liu et al. 2012a; Zhao et al. 2008a). Ft1-4 (Ft homolog), Dchs1-2 (Ds homolog), and Fjx1 (Fj homolog) are known to regulate planar cell polarity; however, their connection to other Hippo pathway components still needs to be explored (Brittle et al. 2010; Hiemer and Varelas 2013; Skouloudaki et al. 2009; Sopko et al. 2009; Zhao et al. 2007).

The other downstream components like Dco and Lowfat homolog have not been shown yet to function within the Hippo pathway (Sopko et al. 2009; Zhang et al.

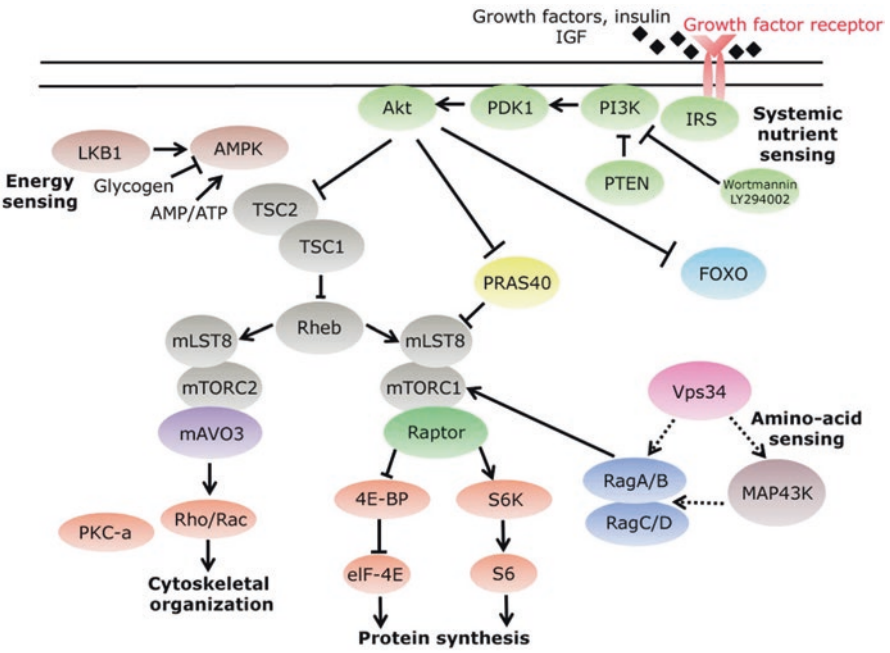

Fig. 4 Hippo pathway is linked to many biological and developmental processes. Hippo signaling has been shown to participate in generating myriad cellular responses that are aimed at attaining tissue homeostasis in addition to regulating organ size. Thus, the role of Hippo signaling is implicated not only during organ development but also in differentiated tissues. Further, tumorigenesis has also been attributed to dysregulation of Hippo signaling pathway placing it in the global network of regulatory mechanisms required for proper growth

2008a, 2011a; Zhao et al. 2010a). However, Dco homolog CK1δ/ε has been shown to be involved in YAP/TAZ degradation (Zhao et al. 2010b). Neurofibromatosis type II (NF2), the Mer homolog, is the most extensively studied upstream regulator in mammals (Sekido 2011; Striedinger et al. 2008; Zhang et al. 2009b; Zhao et al. 2007). NF2 interacts with CD44 and adherens junction to relay the signal downstream to other Hippo pathway components during contact inhibition (Li et al. 2012; Morrison et al. 2001; Zhao et al. 2007). KIBRA is known to interact with Lats2 to promote its phosphorylation (Zhang et al. 2012). It also protects Lats2 from proteosomal degradation by preventing its ubiquitination. KIBRA is also the transcriptional target of Hippo pathway (Angus et al. 2012; Ishiuchi and Takeichi 2012; Visser-Grieve et al. 2012; Xiao et al. 2011). Angiomotin family (AMOT) interacts with its PPxY domain to YAP WW domain and TAZ PDZ domain independent of the upstream components. This interaction inhibits the activity of YAP/TAZ (Chan et al. 2011; Paramasivam et al. 2011; Skouloudaki and Walz 2012; Wang et al. 2009, 2012a; Zhao et al. 2011a). Ex1/FRMD6/Willin (Ex homolog) interacts with upstream Hippo pathway components like Mer (Angus et al. 2012; Ishiuchi and Takeichi 2012; Visser-Grieve et al. 2012). Crb interacts with YAP/TAZ and promotes its phosphorylation, which is

dependent on cell density and at the same time inhibits TGF-β SMAD pathway (Varelas et al. 2010b). Unlike *Drosophila* RASSF1, mammalian RASSF homologs activate MST1/2 (Avruch et al. 2012; Guo et al. 2007; Hergovich 2012; Hwang et al. 2007; Kim et al. 2003; Polesello et al. 2006; Ribeiro et al. 2010; Schagdarsurengin et al. 2010; Seidel et al. 2007).

NPHP4, a known cilia-associated protein that is mutated in the severe degenerative renal disease nephronophthisis, acts as a potent negative regulator of mammalian Hippo signaling (Habbig et al. 2011, 2012). NPHP4 directly interacted with the kinase Lats1 and inhibited Lats1-mediated phosphorylation of the Yes-associated protein (YAP) and TAZ (transcriptional coactivator with PDZ-binding domain), leading to derepression of these protooncogenic transcriptional regulators. Moreover, NPHP4 induced release from 14-3-3 binding and nuclear translocation of YAP and TAZ, promoting TEA domain (TEAD)/TAZ/YAP-dependent transcriptional activity (Habbig et al. 2011). ITCH interacts with LATS to negatively regulate its stability (Ho et al. 2011; Salah et al. 2011; Wang et al. 2012a). α-Catenin interacts with YAP and affects its stability by stabilizing the YAP/14-3-3 complex to restrict YAP activity and by preventing PP2A to interact with YAP (Azzolin et al. 2012; Schlegelmilch et al. 2011; Silvis et al. 2011; Tsai et al. 2012) (Varelas 2010 #1830; Konsavage 2013 #3450; Mauviel et al. 2012 #3755). Zona occludens-2 (ZO-2) promotes the pro-apoptotic function of YAP (Oka et al. 2010). The ASPP (apoptosis-stimulating protein of p53) family of proteins can function in the nucleus to modulate the transcriptional activity of p53, with ASPP1 and ASPP2 contributing to the expression of apoptotic target genes (Vigneron et al. 2010). ASPP increases YAP/TAZ nuclear availability by preventing LATS interaction with YAP/TAZ (Vigneron et al.). Similarly, PP1A interacts with ASPP1 to dephosphorylate TAZ leading to increased TAZ nuclear availability (Liu et al. 2010, 2011).

In mammalian cell lines, E-cadherin acts as an upstream regulator of the pathway, which activates the pathway in response to contact inhibition. YAP and TAZ interact with several transcriptional factors. YAP/TAZ interacts with TEAD1/4 and Runx2. TAZ interacts with thyroid transcription factor-1, peroxisome proliferator-activated receptor gamma (PPARγ), Tbx5, Pax3, and Smad2/3/4. Yap interacts with p73 to mediate its pro-apoptotic functions. Various target genes are as follows: *CTGF*, *AREG*, *BIRC5-2*, and *GLI-2* (Liu et al. 2012b; Zhao et al. 2008a, 2010a). YAP1 interacts with sonic hedgehog pathway to promote the proliferation of cerebellar granule neuron precursors (CGNPs). TAZ inhibits Wnt signaling by inhibiting the phosphorylation of dishevelled (DVL) by CKIδε. YAP/TAZ has also been shown to interact with SMAD to regulate tumorigenesis (Zhang et al. 2011a; Zhao et al. 2011b).

The Insulin Receptor Signaling Pathway: Regulation of Cell Size

The pin-head screens showed a large number of mutations that primarily caused decreased growth due to formation of smaller cells (Oldham et al. 2000a; Stocker and Hafen 2000). These mutants were subsequently categorized into two well-

studied signaling pathways: the insulin/phosphoinositide 3-kinase (PI3K) pathway and the TOR (target of rapamycin) pathway. Using genetic and biochemical strategies, the epistatic and molecular interactions were elucidated for genes that comprise these pathways.

The Regulation of Cell Size and Not Cell Numbers

The PI3K Pathway

Drosophila has one insulin/IGF (insulin-like growth factor) receptor homolog known as dInR (Chen et al. 1996; Fernandez et al. 1995) and several insulin-like peptides (dILPs) (Brogiolo et al. 2001). These together control the carbohydrate metabolism and growth in flies (Ikeya et al. 2002; Rulifson et al. 2002). Through a mechanism that involves phosphorylation of its carboxy-terminal end, the dINR recruits downstream signaling molecules without the need for adaptor proteins. The signaling also involves the insulin receptor substrate (IRS) protein Chico, which contains a phosphotyrosine-binding domain (PTB) that facilitates its binding with activated dINR (Bohni et al. 1999; Poltilove et al. 2000). Subsequently, the pathway functions by activating the PI3K pathway, via activation of the *Drosophila* PI3K-Dp110 and its adaptor subunit Dp60 (Leevers 2001; Leevers et al. 1996; Weinkove et al. 1999). Dp110/Dp60 heterodimers are recruited to the plasma membrane following the binding of p60 SH2 domain to phosphorylated dInr and Chico, which allows the PI3K access to the phosphoinositide substrates in the plasma membrane. This sets up a signaling cascade in which PIP3 transduces the signal to downstream effectors that contain the PIP3-binding PH domains and causes relocalization of these proteins to the plasma membrane (Fig. 5).

In flies, two such effectors exist—which are the *Drosophila* homolog of phosphoinositide-dependent kinase 1 (PDK1) and its substrate AKT aka protein kinase B (PKB). PDK1 localizes to the membrane during low levels of PI3K activity via its affinity to PIP3, whereas AKT requires high levels of PI3K activity to become membrane localized, through a process involving binding of PIP3 to its PH domain and phosphorylation by PDK1 (Vanhaesebroeck and Alessi 2000). In flies, the activity of DAkt is reduced in the absence of Dp110, and co-expression of dPDK1 and dAKT activates dAKT and induces growth (Cho et al. 2001; Radimerski et al. 2002b; Rintelen et al. 2001) (Fig. 5).

A negative regulator of the PI3K activity is the lipid phosphatase PTEN, which removes the 3′ phosphate from three phosphoinositides generated by PI3K (Gao et al. 2000; Goberdhan et al. 1999; Huang et al. 1999) (Fig. 5). Genetic interaction studies support the model where PTEN directly antagonizes PI3K. Loss of PTEN leads to overgrowths due to increased levels of PIP3 (Oldham et al. 2002). Recently, the FOXO family of transcription factors was identified as the target that enabled AKT to regulate growth (Tran et al. 2003). AKT-mediated phosphorylation of FOXO antagonizes its transcriptional activity by creating a 14-3-3 binding site that leads to

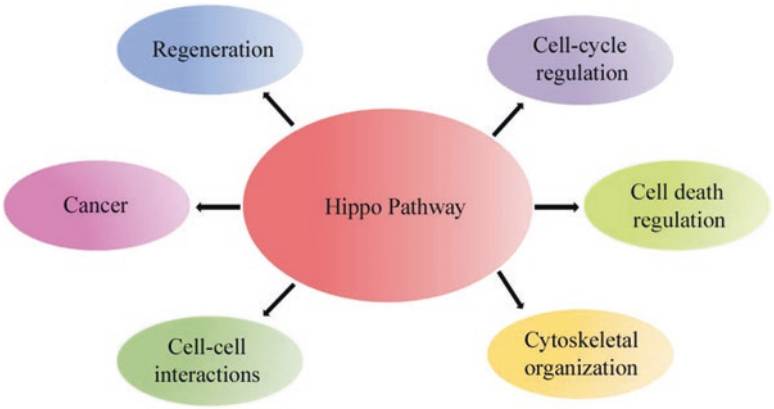

Fig. 5 Model depicting regulation of INR/TOR signaling pathway governed by nutritional status in *Drosophila*. Cellular growth in part is also dependent on the availability of nutrients. This aspect of growth regulation is mainly regulated by the insulin/TOR signaling pathway. Some of the well-studied players of the pathway include phosphatidylinositide 3-kinase and Akt that integrate upstream signaling from growth factor receptors and relay it to TSC1 and TSC2 to regulate ribosomal and protein biosynthesis in addition to actin organization. Other energy-sensing and amino acid-sensing mechanisms are also thought to interact with the core TSC/TOR pathway. However, the exact role or the mechanism by which this takes place remains largely unknown

cytoplasmic sequestration of FOXO (Brunet et al. 1999, 2002; Burgering and Kops 2002). *Drosophila* has one FOXO family transcription factor (dFOXO)—which functions downstream of AKT. Interestingly, loss of function of dFOXO has no apparent effect on cell size or growth as flies homozygous mutant for dFOXO are viable and normal in size (Junger et al. 2003).

The loss of function of Dp110, p60, chico, dINR, dPDK1, and dAKT shows similar effects on cell size and tissue growth (Fig. 5). For example, twin-spot analysis revealed that loss-of-function clones of mutations in these genes are smaller than the corresponding wild-type twin clones that lead to formation of smaller structures (Bohni et al. 1999; Brogiolo et al. 2001; Rintelen et al. 2001; Verdu et al. 1999; Weinkove et al. 1999). Overexpression of PI3K pathway components like Dp110 leads to increased insulin/PI3K signaling and a corresponding increase in cell size, cell number, and tissue growth (Goberdhan et al. 1999; Huang et al. 1999; Leevers et al. 1996). Overall, changes in levels of insulin/PI3K signaling have profound effects on organ and organismal size due to effects on cell growth and cell division throughout development and affect the final body/organ size (Fig. 5).

The TSC-TOR Pathway

Two target of rapamycin (TOR) genes, *TOR1* and *TOR2*, were initially identified in yeast and were shown to be kinases that regulate growth in all organisms by acting as nutrient sensors that couple signaling to nutrient availability (for review, see

Neufeld 2003; Gingras et al. 2001). *Drosophila* TOR (dTOR) promotes growth by stimulating translation via promoting the activity of the *Drosophila* S6Kinase (Montagne et al. 1999) and inhibiting the *Drosophila* 4E-BP1 (a homolog of the eukaryotic translation initiator 4E)—the translational inhibitor of eIF4E, which is a part of the translation initiation complex (Gingras et al. 2001; Lasko 2000). Hyperphosphorylation of d4E-BP1, which is in part controlled by the TOR kinase, relieves its interaction with eIF4E leading to translation initiation.

TOR signaling is negatively regulated by a complex formed by the tuberous sclerosis complex tumor suppressors, TSC1 and TSC2 (Marygold and Leevers 2002) (Fig. 5). Mutations in TSC1/2 cause formation of large cells and are implicated in the inherited benign hamartomas observed in the tuberous sclerosis patients (Kandt 2002; Montagne et al. 2001). The *Drosophila Tsc1/2* genes show similar effects on cell size and were identified by several groups in the *eyFLP cell lethal* screens as mutants with overgrown heads (Gao and Pan 2001; Potter et al. 2001; Tapon et al. 2001). Loss of *Tsc1/2* causes increased growth, whereas overexpression of TSC1/2 causes reduced growth due to slow cell cycle progression in the mutant cells. Growth regulation via TSC1/2 happens through preventing dS6K activation via dTOR (Gao et al. 2002; Radimerski et al. 2002a, b). Another important component of this pathway is the GTPase *Rheb*, which is a target of TSC (Saucedo et al. 2003; Stocker et al. 2003; Zhang et al. 2003). The Rheb-GTP levels play a central role in regulating the activity of TOR pathway and the TOR protein that exists in two large multimeric complexes in the cell, viz., the rapamycin-sensitive TORC1 complex and the rapamycin-resistant TORC2 complex (Hara et al. 2002; Kim et al. 2002, 2003; Loewith et al. 2002; Sarbassov et al. 2004).

The TORC1 complex consists of TOR, Raptor, and LST8; and responds to the presence of growth factors and nutrients to control protein synthesis (Fig. 5). The small GTPase protein Rheb (Ras homolog enriched in the brain) is a direct activator of TORC1 (Long et al. 2004; Saucedo et al. 2003; Stocker et al. 2003), and the tuberous sclerosis (TSC) complex (TSC1/TSC2) negatively regulates TORC1 by functioning as a GTPase-activating protein (GAP) for Rheb (Potter and Xu 2001; Zhang et al. 2003). Growth factors such as insulin or insulin-like growth factors (IGFs) activate TORC1 signaling upstream of the TSC1/TSC2 (TSC1/2) complex through the insulin receptor (InR)/phosphoinositide 3-kinase (PI3K)/AKT signaling pathway (Inoki et al. 2002; Potter et al. 2002). TORC1 also senses nutrient availability. Amino acids regulate TORC1 through mechanisms independent or downstream of TSC complex, and recently the Rag small GTPases have been shown to interact with TOR and promote TORC1 activity by controlling its subcellular localization (Nellist et al. 2008; Sancak et al. 2010).

TORC2 complex (Fig. 5) consists of TOR, Rictor, Sin1 (stress-activated map kinase-interacting protein 1), and LST8 and phosphorylates and activates several AGC family kinases, including AKT, serum and glucocorticoid-regulated kinase (SGK), and protein kinase C (PKC), and thereby regulates cell survival, cell cycle progression, and metabolism (Pearce et al. 2010) (Li 2010 #8573; Gao 2010 #8574). In contrast to TORC1, little is known about the upstream activators of mTORC2. Although the general mechanisms have not been accepted, PI3K, TSC, and Rheb

have been shown to regulate TORC2 activity, and Rictor has been identified as a substrate of S6 kinase (S6K), suggesting possible regulation of TORC2 through the TORC1 pathway (Dibble et al. 2009; Treins et al. 2010; Yang et al. 2006). Nevertheless, it is generally thought that growth factors may control TORC2, either directly or indirectly (Zinzalla et al. 2011). TORC2 has been proposed to function independent of amino acid availability (Jacinto et al. 2006); however, recent findings show that amino acids may also activate TORC2 (Tato et al. 2011).

The central role of TOR in cell growth has been largely attributed to TORC1, but mounting evidence points to a role for TORC2 as well in this basic cellular process. For instance, TORC2 localizes in polysomal fractions and associates with ribosomal proteins, indicating a potential role for TORC2 in protein synthesis and maturation (Cybulski and Hall 2009; Zinzalla et al. 2011). *lst8* knockout flies are viable but small, similar to *rictor* mutants but dissimilar to files with *tor* or *rheb* mutations, which are lethal (Avruch et al. 2009; Liao et al. 2008; Wang et al. 2012b). Neither loss nor overexpression of LST8 affected the kinase activity of TORC1 toward S6K or autophagy, whereas the kinase activity of TORC2 toward AKT was completely lost in the *lst8* mutants (Avruch et al. 2009; Liao et al. 2008; Wang et al. 2012b).

In terms of effects of TOR signaling on growth phenotypes in *Drosophila*, loss of dTOR leads to a decrease in larvae size; however, the larvae fail to mature and die before reaching adulthood. In mosaic *Drosophila*, loss of dTOR leads to a decrease in cell size while maintaining the general organization of the tissue (Oldham et al. 2000b; Zhang et al. 2000). However, it is less clear how cell size is regulated downstream of mTOR. One of the most potent candidates in this regulation is S6K. In *Drosophila*, knockout of *S6K* results in high rates of embryonic lethality. In the surviving adults, however, there is a decrease in body size. Knockdown of either dPTEN or dTSC1is sufficient to increase cell size; however, a double knockdown of dPTEN and dTSC1 has additive effects on cell size regulation. This suggests that in *Drosophila*, the pathways may have independent components in the regulation of cell size (Gao and Pan 2001). It may also highlight the differences in the regulation of TSC2 by AKT in *Drosophila* as seen by mutations of the AKT phosphorylation sites on TSC2 (Dong and Pan 2004; Pan et al. 2004). Loss of either dPTEN or dTSC1 can lead to increases in cell size; however, a report has suggested that only knockdown of dTSC1 leads to increases in dS6K (Radimerski et al. 2002a), whereas other reports have also seen increases in dS6K with the knockdown of dPTEN (Sarbassov et al. 2004; Yang et al. 2006). It is possible that dTSC1 regulates cell size in a dTOR-dependent manner, whereas dPTEN partially regulates cell size in a dTOR-independent manner (Radimerski et al. 2002b).

In conclusion, the TOR signaling pathway is a complex network of cell size regulators that is also implicated in tumorigenesis and cell survival (Fig. 5). Several pathways interact and intersect with the TOR pathway at multiple points upstream and downstream of TOR.

Growth Regulation: A Network of Tumor Suppressors

Overall, growth control occurs through the Hippo and TSC-TOR pathways in conjunction with pathways regulating pattern formation during development. These pathways intersect in complicated signaling networks in all cell types and coordinately regulate overall growth of an organism. Our progress in understanding of these pathways has led the way to find molecules and interactions important for regenerative growth and wound healing—phenomena that have been well documented but not well understood at the molecular level for a long time. In addition, the establishment of these growth regulatory networks has led many insights in the fields of cancer (e.g., the underlying genetics and biology link between hamartomas and TSC genes; schwannomas and NF2; YAP and hepatocellular carcinoma, TAZ and breast cancer, etc.). In the future, it will be interesting to learn about the regulation of these pathways by extracellular and intracellular mechanisms, an area expected to expand rapidly with our increased understanding of the integration points in the circuitry of these networks.

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Drosophila Cancer Modeling Using the Eye Imaginal Discs

Karishma Gangwani, Kirti Snigdha, Mardelle Atkins, Shree Ram Singh, and Madhuri Kango-Singh

Introduction

Cancer results from the accumulation of genetic defects that drive key cellular processes like unrestrained cell growth and proliferation, apoptosis, and changes in metabolic pathways. Key genes whose mutation promotes the development of cancer are largely grouped into two classes: oncogenes and tumor suppressors. Oncogenes are typically activated by mutation, and their activity promotes cell growth and survival. Proto-oncogenes (e.g., Ras or Myc) function as components of signaling cascades and are deregulated in >25% of human tumors (Samatar and Poulikakos 2014). Tumor suppressors are genes whose mutational loss allows the survival and uncontrolled proliferation of otherwise damaged or aberrant cells (Hanahan and Weinberg 2011; Hariharan and Bilder 2006). They include genes such as *RB* (retinoblastoma-associated) and *TP53* genes (Duronio and Xiong 2013). Cancer cells additionally can evade apoptosis or cell death by increasing the activity

K. Gangwani · K. Snigdha
Department of Biology, University of Dayton, Dayton, OH, USA

M. Atkins
Department of Biological Sciences, Sam Houston State University, Huntsville, TX, USA

S. R. Singh
Mouse Cancer Genetics Program, National Cancer Institute, Frederick, MD, USA

M. Kango-Singh (✉)
Department of Biology, University of Dayton, Dayton, OH, USA

Center for Tissue Regeneration and Engineering at Dayton (TREND), University of Dayton, Dayton, OH, USA

Premedical Program, University of Dayton, Dayton, OH, USA

Integrative Science and Engineering Center (ISE), University of Dayton, Dayton, OH, USA
e-mail: mkangosingh1@udayton.edu

A. Singh, M. Kango-Singh (eds.), *Molecular Genetics of Axial Patterning, Growth and Disease in Drosophila Eye*,
https://doi.org/10.1007/978-3-030-42246-2_9

of anti-apoptotic genes (*bcl-2*, *bcl-xL*, *bcl-w*) and of pro-survival factors (*igf-1*, *igf-2*, *survivin*, *xiap*, *birc5*, etc.) or by downregulating the action of pro-apoptotic genes (*Bax*, *PUMA*, *Bin*) (Hanahan and Weinberg 2011).

The sequencing of the *Drosophila* genome led to the realization that approximately 70% of human disease-associated genes have a single *Drosophila* homolog (Reiter et al. 2001; Yamamoto et al. 2014; Adams et al. 2000; Rubin and Lewis 2000). Subsequent comparative genomic studies have highlighted the relevance of this model organism to study the function of conserved genes and their roles in human diseases (Wangler et al. 2015). The conservation of genes and genetic pathways, especially oncogenes, tumor suppressor genes, and pro- and anti-apoptotic genes, and proliferation machinery in *Drosophila* provide a distinct advantage for studying gene regulation and the effects of mutations on cancer growth particularly during the early stages of cell transformation. Furthermore, metabolic cell reprogramming seems to be conserved, as a role for ROS in cancer growth and progression has been identified as well as similar mechanisms underlying shifts in tumor glucose metabolism in both flies and humans (Perez et al. 2017; Diwanji and Bergmann 2018; Herranz and Cohen 2017; Eichenlaub et al. 2018; Wang et al. 2016). Additionally, flies provide a vast array of genetic tools for manipulation of gene expression, for knocking down gene expression in a tissue-specific manner, and for testing potential inhibitors of cancers. This is coupled with a rapid life cycle, low gene redundancy, and well-characterized developmental pathways. Thus, *Drosophila* is an important preclinical model for cancer studies and has consistently provided insights into the signaling pathways, genes, and cell behaviors that drive tumorigenesis in humans (Wangler et al. 2015).

Classically, the limitations of the *Drosophila* tumor models have appeared to be the lack of an elaborate closed circulatory system which plays a key role in the metastasis of cancer cells from the site of the primary tumor to distant organs and in tumor angiogenesis; also *Drosophila* lack an adaptive immune system. However, in *Drosophila*, the initial steps of metastasis are conserved as cells must escape their epithelia by dissolving their local extracellular matrix, surviving extrusion, and undergoing an EMT-like process, permitting modeling of these early, critical steps. Recent studies have also found that *Drosophila* tumors also undergo a similar process to angiogenesis (Grifoni et al. 2015). Finally, while lacking an adaptive immune system, flies have a robust innate immune system, and a role for macrophages in inflammatory signaling in the tumor microenvironment has been characterized (Cordero et al. 2010; Ratheesh et al. 2015; Pastor-Pareja et al. 2008). Thus, with continued study, we have found that while the systems appear grossly different, consistently we find that similar cellular and molecular changes drive the tumor development processes.

Historically, the compound eye of *Drosophila* is a proven model for studying many diseases despite the noticeable anatomical differences between insect and vertebrate eyes. The *Drosophila* eyes develop from the larval eye imaginal discs and form the eyes, antenna, and the dorsal head cuticle in the adult (Wolff and Ready 1991; Cagan and Ready 1989; Salzer and Kumar 2010; Kumar 2011). Each unit eye or ommatidium is a repeating pattern of a dozen cell types arranged in a hexagonal

array that enhances visual acuity of the fly (Kumar et al. 2012). Traditionally, the eye imaginal discs have been an ideal model to study developmental mechanisms that govern patterning, the regulation of the cell cycle, growth control, programmed cell death, compartment boundaries, cell fate specification, and planar cell polarity. Importantly, if the eyes are ablated, flies can survive, facilitating the use of the eye tissue for disease modeling. One of the first examples of this was the isolation of a mutant fly strain called *eyeless* (*ey*^{1}) that lacked eyes but is otherwise completely normal (Hoge 1915). Other mutants that showed eye development defects were also identified (Pignoni et al. 1997; Jang et al. 2003; Bonini et al. 1993; Cheyette et al. 1994; Mardon et al. 1994; Czerny et al. 1999). The characterization of *ey*^{1} and other similar mutants generated an extensive knowledge of eye development through larval and pupal metamorphosis. Of the different model systems developed in *Drosophila*, the eye imaginal discs are ideal models of choice for cancer studies because of the tight, well-characterized regulatory mechanisms that control cell proliferation, cell fate, and patterning in the eye. Any dysregulation from these controls is easily detectable and quantifiable. Thus, the depth of knowledge, combined with the vast array of genetic tools found in *Drosophila*, makes the fly eye imaginal discs an ideal model system for studying not only development but also complex diseases like cancer. This is particularly true for the initial cell biological changes that promote cancer growth and cell transformation, which can be difficult to assess in more complex models or in culture.

Benign and Neoplastic Tumors in Drosophila

Drosophila has conserved tumor suppressor genes and oncogenes and a plethora of signaling mechanisms that work in a conserved manner from flies to humans. Spontaneous mutants of tumor suppressor genes or oncogenes have been recovered that cause uncontrolled proliferation leading to hyperplastic (benign) and neoplastic (metastatic) tumors. Mutations in the gene *lethal giant larvae* (*lgl*) were first identified in the 1930s that caused neoplasia in *Drosophila* (Hadorn 1937; Gateff and Schneiderman 1969; Mechler et al. 1985). The phenotypes of the mutant larvae included abnormal overproliferation of tissues including the brain, imaginal discs, and hematopoietic organs (Gateff and Schneiderman 1969). The neoplasms grew quickly in a cell-autonomous manner, the epithelial cells showed loss of apicobasal polarity, and the transformed tissue invaded (metastasized) into neighboring regions. Transplantation experiments have further shown that these neoplastic cells can successfully colonize another organism (adult fly) and form tumors, indicating unlimited replicative potential of these cells (Gateff and Schneiderman 1969; Muzzopappa et al. 2017a; Figueroa-Clarevega and Bilder 2015). More recently, other genes such as *discs large* (*dlg*) and *scribble* (*scrib*) were also identified that generate neoplastic tissues due to disrupted apicobasal polarity of epithelial cells (Bilder 2004; Froldi et al. 2010). Biochemical studies showed that Dlg and Scrib proteins colocalized in cells at the septate junctions (corresponding to the tight junctions in vertebrates),

partially overlapping with Lgl (Humbert et al. 2003). An interesting attribute of *Drosophila* tumor suppressor/oncogenic mutants is the propensity of larvae to enter an extended larval phase (i.e., they do not molt or undergo metamorphosis) in response to impaired ecdysone signaling. These mutant larvae lose body fat and form large neoplastic tumors (Brumby and Richardson 2003; Tipping and Perrimon 2014). These characteristics allow for quick and easy detection of mutant larvae and a systematic characterization of associated developmental, metabolic, and functional defects. Overall, these mutants not only shared a common phenotype, a loss in the apical/basal polarity that led to deregulated tissue growth, they also colocalized at the basal septate junctions suggesting that they function in a polarity-regulating pathway (Bilder et al. 2000).

In multicellular organisms, epithelial cells must retain correct cell polarity to maintain proper tissue integrity (Riddiford et al. 2003). In mice, Lgl1-null mutants exhibit severe brain dysplasia due to increased numbers of progenitor cells that fail to differentiate (Klezovitch et al. 2004). In humans, the ortholog of Scribble is a target of downregulation by human papilloma virus in reproductive cancers (Nakagawa et al. 2000). Furthermore, its interactor, HUGL-1, a human ortholog of *lgl*, is downregulated in human cancers such as breast, lung, prostate, ovarian, and colorectal cancers (Grifoni et al. 2004, 2007; Kuphal et al. 2006; Schimanski et al. 2005). We now know that altering cell polarity regulators can lead to epithelial-to-mesenchymal transformation (EMT) to accelerate cancer invasion and metastasis, as well as stem cell-like properties and chemoresistance of cancer cells (Hanahan and Weinberg 2011; Fischer et al. 2015; Ye and Weinberg 2015; Zheng et al. 2015). Together, these results suggest parallel mechanisms for tumor suppression across species.

Since the initial discovery of Lgl, many more tumor suppressors and oncogenes have been identified in *Drosophila* including the initial discovery of the Hippo pathway, which we now know is commonly deregulated in human cancers. Studies by Bryant et al. were some of the first that showcased the *lethal(2)giant discs* (*l(2)gd*) mutant flies as models of a tumor suppressor in *Drosophila* (Bryant and Schubiger 1971). Soon, other tumor suppressor genes like *lethal(2) fat* (*l(2)ft*) (Bryant et al. 1988; Mahoney et al. 1991) and *expanded* (*ex*) were shown to function as tumor suppressor genes as well. Another set of tumor suppressor genes which maintain tissue homeostasis by regulating cell survival and cell death were subsequently identified and found to comprise a previously unknown signaling pathway that regulates growth, now called the Hippo signaling pathway, of which *ex* and *ft* are members (Stern and Bridges 1926; Boedigheimer et al. 1993; Boedigheimer and Laughon 1993). Dysregulation of the pathway leads to benign hyperplasia. The Hippo pathway is highly conserved, and named for the "big-headed" phenotype of mutants isolated from genetic screens in flies, caused by tissue overgrowths and extra interommatidial cells in the pupal retina. These mutations were found to belong to three key genes, *warts* (*wts aka large tumor suppressors*, *lats*), *salvador* (*sav* aka *sharpei*, *shrp*), and *hippo* (*hpo* aka *Drosophila mammalian Ste-20 kinase*, *dMst*). The characterization of these phenotypic defects showed that these genes possess the rare ability to promote proliferation and suppress apoptosis simultaneously

(Edgar 2006). The Hippo pathway gained tremendous attention when Yorkie (Yki, *Drosophila* homolog of mammalian YAP/TAZ) was identified in a yeast two-hybrid screen for Wts binding proteins as a target of the serine threonine kinases Hpo and Wts (Huang et al. 2005; Oh and Irvine 2008; Snigdha et al. 2019). Yki overexpression or activation caused overgrowth phenotypes similar to *sav*, *hpo*, and *wts* loss of function suggesting that Yki is an oncogene. Biochemical and genetic studies in *Drosophila* revealed that Yki is required for normal tissue growth and its activity is inhibited by Wts-mediated phosphorylation (Wu et al. 2003; Dong et al. 2007; Zhao et al. 2008). Since its discovery, and as in human tumors, several *Drosophila* tumor models have discovered a central role for Yki activation in promoting tumor growth. These include the epithelial tumor models of oncogenic cooperation where activation of oncogenes in polarity-deficient cells (e.g., Ras^{V12} $scrib^{-}$) results in increased Yki activity which is required for aggressive tumor growth (Brumby and Richardson 2003; Uhlirova et al. 2005; Suijkerbuijk et al. 2016). Similarly, models of intestinal adenomas ($APC^{-/-}$ cells) show increased Yki activity that promotes tissue growth (Suijkerbuijk et al. 2016; Hall et al. 2010; Chen et al. 2012). Thus, the identification of tumor suppressor and oncogenes in *Drosophila* facilitated not only an understanding of growth regulation, but it also led to the identification of signaling and cell-cell interactions that promote tumor growth.

Oncogenes such as RAS are among the most frequently mutated genes in human cancers, and tumors harboring activating RAS mutations (e.g., Ras^{V12}) are among the most difficult to treat (Stephen et al. 2014). To understand its role, Karim and Rubin expressed the oncogenic *Drosophila* RAS isoform $dRas1^{G12V}$, mimicking the most common human mutation, in developing imaginal discs using the GAL4-UAS-based misexpression system (Karim and Rubin 1998). They found that overexpression of $dRas1^{G12V}$ caused cell autonomous hyperplasia but also induced cell death away from $dRas1^{G12V}$-expressing cells, a form of compensatory apoptosis. Loss-of-function mutations in *raf*, *mek*, and *mapk* dominantly suppressed these phenotypes suggesting the necessity of the MAPK pathway in promoting hyperplasia. Using the FLP/FRT system (Golic and Lindquist 1989), to generate small homozygous clones, Richardson and colleagues observed similar transformation phenotypes (Brumby and Richardson 2003). However, the understanding of how Ras signaling functions in normal cells, as well as the identification of many of its key components, originally occurred due to studies on *Drosophila* eye imaginal disc development (Karim et al. 1996). Previously, the *Drosophila* Sevenless (Sev) and Boss (Bride of Sevenless) receptor/ligand pair were identified as key regulators of eye development, specifically for the specification of the R7 photoreceptor in the compound eye, which initiates neural development through receptor tyrosine kinase (RTK)-mediated inductive signaling (Tomlinson and Ready 1987; Cagan et al. 1992). Genetic analysis of Sev and Boss, and screens for modifiers of the effects of gain or loss of Sev or Boss, led to the identification of the components of this RTK signal transduction pathway including Ras, Son of Sevenless (Sos, a guanine nucleotide releasing factor), downstream of receptor kinases (Drk, a SH2 domain containing adaptor protein), Raf, and other mitogen-activated protein kinases (MAPKs) as well as the ETS-domain transcription factor effectors *pointed*, *capicua*, and *yan*

(Hafen et al. 1993; Wassarman et al. 1995; Klambt 1993; Lai and Rubin 1992; Tseng et al. 2007; Jimenez et al. 2000). Thus, studies such as these helped established the key roles of RAS-MAPK signaling pathway in regulating proper tissue growth and differentiation and in promoting transformation.

The Ras signaling pathway is upregulated in many human cancers, but increased Ras signaling alone is not sufficient to induce malignant tumors. The relative ease in *Drosophila* of conducting screens to identify genetic interactions has thus been key to our understanding of how Ras activation can actually promote malignancy (Dominguez and Hafen 1997). For example, the Ras pathway is artificially activated in the developing eye epithelium by reducing activity of Ksr (Huang and Rubin 2000), an interesting Ras effector identified as a genetic modifier of Ras activity in flies and worms (Kornfeld et al. 1995; Sundaram and Han 1995; Therrien et al. 1995). Huang and Rubin used the overexpression "EP" system (Rorth 1996; Rorth et al. 1998) to screen genes for the ability to alter the resulting hyperplasia of *ksr* mutants. Their "genetic modifier screen" successfully identified four enhancers and eight suppressors of the hyperplastic phenotype, including Lk6, a kinase downstream of MAPK that has proven a significant tumor suppressor (Huang and Rubin 2000; Proud 2007). More recently, using the eye-specific "FLP-out" system in *Drosophila*, Zoranovic et al. (2018) have shown that the tetraspanin family member Tsp29Fb regulates EGFR signaling and epithelial architecture and restrains tumor growth and invasion (Zoranovic et al. 2018). Using their eye model, they experimentally confirmed Tsp29Fb as a key regulator of EGFR/Ras-induced epithelial tumor growth and invasion. Tsp29Fb functions as a tumor suppressor by inhibiting Ras signaling and by maintaining epithelial cell polarity (Zoranovic et al. 2018). EGFR is still needed in RAS-dependent tumors because of an Arf6/Hh circuit. ADP-ribosylation factor 6 (Arf6) regulates endocytosis, vesicle transport, and secretion. It is promoted by EGFR and controls Hedgehog (Hh) signaling by regulating Hh cellular trafficking. Blocking EGFR or Arf6 results in inhibition of Hh and suppresses growth driven by oncogenic Ras mutations in fly and human tumor cells (Chabu et al. 2017). These studies emphasize the power of loss-of-function and gain-of-function screens to identify oncogenes and tumor suppressors in *Drosophila*, providing an opportunity to explore these factors with single-cell resolution and to place them into cancer networks in situ (Karim and Thummel 1991; Layalle et al. 2008; Yan and Perrimon 2015; Shimell et al. 2018).

In summary, the *Drosophila* eye discs have proven very informative not only for dissecting developmental roles of growth controlling tumor suppressor and oncogenes but have provided an excellent tissue model for studying alterations that promote tumor growth and neoplastic invasion. In the subsequent sections, we describe the techniques generally employed for eye-specific tumor models in *Drosophila*, the different *Drosophila* cancer models, and the recent advances in cancer biology from studies in *Drosophila*.

The Genetic Toolkit: The MARCM System and Tissue-Specific Drivers in *Drosophila*

Genetically defined tumors of varying malignancy can be reproducibly engineered in *Drosophila* by introducing gain- and loss-of-function mutations in a subset of progenitors in an otherwise wild-type tissue using the MARCM technique (Lee and Luo 2001; Morata and Ripoll 1975). The MARCM tool combines yeast-derived FLP/FRT (FLP recombinase/FLP recognition target)-mediated mitotic recombination (Xu and Rubin 1993) with FLP-out (Struhl and Basler 1993) and Gal4/UAS (upstream activation sequence) (Brand and Perrimon 1993) target gene expression systems. Using the MARCM method, the expression of any UAS-based transgene, including oncogenes or fluorescent protein cDNAs or inverted DNA repeats for dsRNA-induced gene silencing, is restricted to a clone of cells that have lost a specific genetic locus and the Gal80 repressor due to recombination (Luo et al. 2006). The combined use of Gal80 and the Gal4/UAS system is an extremely useful addition to the *Drosophila* genetic toolkit, especially when temporal control of gene expression is an important aspect of mosaic generation. In the context of the MARCM technique (Fig. 1), temporal control of Gal80 (and thus Gal4-regulated gene expression) can be achieved by regulating FLP induction. However, in this case, functional repression by Gal80 can be only released—but not induced—in a temporally controlled manner and only in the daughter cells in which copies of the Gal80 gene have not been inherited (Fig. 1). Gal80 is also useful in FLP-out experiments, since control of its induction is possible. In one variation of the FLP-out method, Gal80 can be constitutively turned on or off in FLP-expressing cells using tub^{P}>stop>Gal80 (FLP-in) or tub^{P}>Gal80>(FLP-out) transgenic constructs, wherein ">" denotes a FLP consensus target site (Zecca and Struhl 2002; Gordon and Scott 2009; Bohm et al. 2010). Temporal regulation can also be achieved by linking Gal80 to promoters that are active at a specific stage of development. Still, inherent in these uses of Gal80 is its perdurance, which prevents rapid release of Gal4 inhibition. Gal80 can perdure as long as 40 h after recombination in MARCM experiments in imaginal discs (de la Cova et al. 2004), so experiments need to be designed with this in mind.

The *eyFLP* MARCM method relies on a MARCM tester stock which expresses the FLP recombinase under the control of a developmental enhancer of the *eyeless* gene (*eyFLP*) (Newsome et al. 2000; Quiring et al. 1994). In this way, GFP-labeled clones are generated in both peripodial and columnar epithelium of the EAD (eye/antenna discs), the neuroepithelium of the brain, and the developing gonads throughout embryonic and larval stages (Newsome et al. 2000). A recent version of the *eyFLP*, referred to as *ey3.5 FLP*, drives recombination only in the EAD making it an eye-specific FLP recombinase driver for generating *eyFLP* MARCM clones (Parks et al. 2004) (Fig. 1). Clones can be easily followed until adulthood as the EAD develops into the adult eye, antenna, and head capsule. Using the eyFLP technique, the Richardson and Xu groups found that a specific combination of defects, namely, disruption of apicobasal cell polarity together with the expression of an

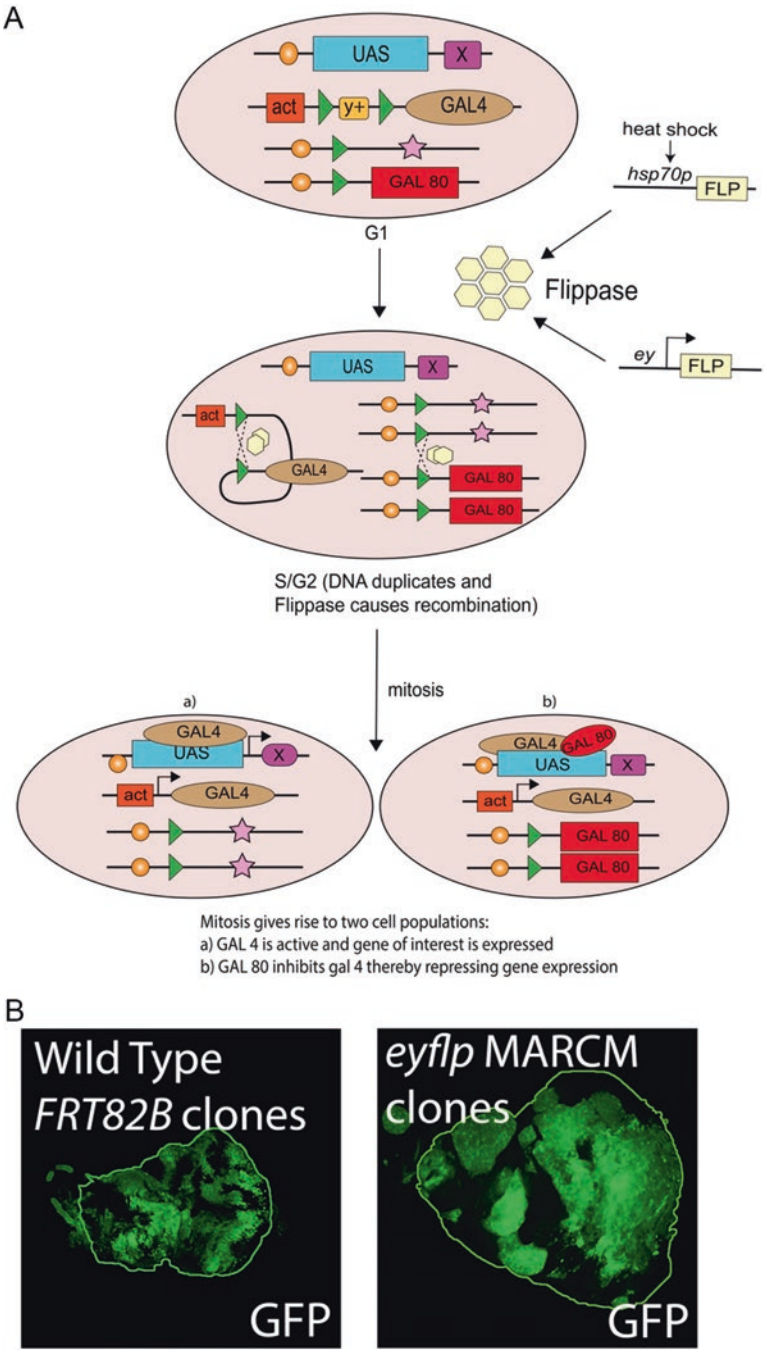

Fig. 1 *Drosophila* tumor modeling using the MARCM system. (**a**) Cartoon showing the genetic basis of the mosaic analysis with a repressible cell marker (MARCM) system. After site-specific

activated version of the oncogenes Ras or Notch, resulted in excessive cell proliferation and metastasis (Brumby and Richardson 2003). Mutant cell clones were then analyzed for cell proliferation and for invasion of distant larval tissues. $Ras^{V12}scrib^{-/-}$ mutant cells hyperproliferate and form secondary tumors in the ventral nerve cord, imaginal tissues, and tracheal branches in the mutant animals. The technique has undergone several modifications and upgrades since then. Now it is possible to positively mark mosaic clones with one or more markers (dual-color MARCM) or combine overexpression systems (e.g., GAL4-UAS with LexA-LexAOP; (Rodriguez et al. 2012)) or use tissue-specific promoters to drive gene expression in specific cells (e.g., Repo Gal4—restrict misexpression in the glia). Furthermore, a widely used method for rapid temporal modulation of Gal4 activity is through a temperature-sensitive Gal80 allele (Gal80ts) that is active and represses Gal4 at 18 °C but is inactive at 29 °C (McGuire et al. 2004). This feature allows fairly tight and reversible temporal control during overexpression or knockdown experiments by a simple shift of the incubation temperature from 18° to 29 °C at any stage of development. The modular nature of these tools and techniques has led to the development of a wide variety of strategies now available for developing preclinical cancer and disease models.

In the sections below, we describe how a variety of cancer models have been developed using the *Drosophila* eye disc as an organ system of choice. It should be noted that while a number of cancer models have utilized the *Drosophila* eye disc, other *Drosophila* cell and tissue types, e.g., brain, intestine, hemolymph, and follicle/germ cells, have also proven useful in modeling cancer.

The Oncogenic Cooperation Model Between Loss of Polarity and Activated Oncogenes

Metastasis consists of multiple steps: growth of the primary tumor, invasion into surrounding stroma, intravasation into blood and lymphatic circulations, extravasation to the secondary organs, and growth of secondary tumors (Fidler 2003; Steeg 2006). Metastasis is thought to occur when multiple mutations accumulate (Kinzler and Vogelstein 1996). For example, in the human colon, tumorigenesis starts with

Fig. 1 (continued) mitotic FRT recombination (green arrowhead) via flippase (yellow hexagon), a heterozygous mother cell can give rise to two daughter cells in which the chromosome arms distal to the recombination site become homozygous. Driven by the actin promoter, GAL80 is ubiquitously expressed in one cell and efficiently suppresses GAL4-dependent expression of a UAS gene. The twin clone daughter cell homozygous for the mutant gene (asterisk) no longer contains GAL80, and thus GAL 4 is active to express the gene of interest (purple X) under UAS control. Therefore, the gene of interest or marker gene can be specifically turned on by GAL4 in homozygous mutant cells. (**b**) Panels show L3 *Drosophila* eye imaginal discs with MARCM clones marked with GFP using *eyeless* flippase system. The left panel shows wild-type FRT82B control clones, while the right panel shows Ras^{V12} $scrib^{-}$ MARCM clones showing tumor growth. The boundary of the eye disc is marked to show the overall area of the disc. GFP negative area denotes wild-type cells

mutations in the *APC* (adenomatous polyposis coli) gene. However, *APC* alteration by itself is not sufficient to cause malignancy; mutations in additional genes such as members of the RAS family are required for cancer progression (Morris et al. 2008). Similarly, using the $dRas1^{G12V}$ "benign" tumor model, Pagliarini and Xu found that reducing activity of cell polarity genes *scrib*, *lgl*, *dlg*, *bazooka* (baz), *stardust* (sdt), or *cdc42* in *Drosophila* EADs enabled $dRas1^{G12V}$-expressing cells to progress toward profound overgrowth, invasiveness, and secondary growth (Pagliarini and Xu 2003). These studies used the cephalic complex which included the eye discs attached through the optic stalk (nerve) to the larval brain to model tumor growth and metastasis. A variety of phenotypic similarities were observed between $dRas^{G12V}$; $scrib^{-/-}$ tumors and human tumors; for example, the basement membrane (BM) was degraded and cells invaded into neighboring tissues, a behavior regulated by E-cadherin (Pagliarini and Xu 2003). These results indicate a key role of maintaining proper cell polarity in preventing metastatic progression of benign cells with oncogenic RAS isoforms. These reports elegantly showed that neoplastic tumors could form by oncogenic cooperation (Brumby and Richardson 2003; Pagliarini and Xu 2003) and demonstrated the effects of second site mutations on Ras-mediated hyperplasia (Pagliarini and Xu 2003). A subsequent metastasis screen was performed for additional mutations that cooperated with Ras^{V12} to form aggressive metastatic tumors and revealed a small number of modifiers/genes, e.g., *deep orange* (*dor*) (Chi et al. 2010). Interestingly, it was also demonstrated that oncogenic Ras can establish cooperative intercellular interactions when Ras^{V12} is activated in a cell adjacent to a polarity defective $scrib^{-}$ cell (Wu et al. 2010a). An exchange of signals (JNK, JAK-STAT) feeds the transformation of activated Ras-expressing cells to highly proliferative neoplastic cells (Wu et al. 2010a). These models elegantly demonstrated the cooperation between apicobasal polarity-regulating genes and activated oncogenes like Ras and revealed a role for the JNK and JAK-STAT signaling pathways in promoting neoplastic tumor growth (Wu et al. 2010a). Subsequent studies have revealed the importance of these findings in terms of conservation of these interactions in mammalian cancer models and human cancer (Sonoshita and Cagan 2017) and generated insights on the changes in gene expression, metabolism, and cell-cell interactions that promote tumorigenesis.

The Oncogenic Cooperation Model Involving the Src Oncogene

In a variety of human cancers including melanoma, breast, and colorectal cancers, SRC family kinases (SFKs) are activated by various cues such as growth factors and cell-cell contact (Yeatman 2004). SFKs are linked to malignant progression of human cancers, and, in particular, their activity is frequently associated with metastatic potential. As such they serve as attractive therapeutic targets, but their precise roles in cancer progression remain to be clarified. Overexpression of the *Drosophila*

SFK orthologs *dSrc42A* and *dSrc64B* causes mild effects on growth and causes a small rough eye phenotype due to apoptosis. However, when coupled with other oncogenes such as Ras, high levels of Src activity can lead to malignant overgrowth and invasion (Vidal et al. 2007). Normally, SFKs contain a negative regulatory C-terminal domain, which is frequently mutated in human cancers. C-terminal SRC kinase (CSK) phosphorylates a key regulatory tyrosine residue within this C-terminal domain, causing a conformational change of the domain to inactivate SRC kinase activity. In *Drosophila*, *Csk* (*dCsk*) also antagonizes *Drosophila Src* (*dSrc*), and reducing dCsk activity led to increased Src activity and, somewhat surprisingly, to increased cell proliferation, which depended upon JNK and JAK-STAT signaling (Read et al. 2004; Stewart et al. 2003). Interestingly, knockdown experiments for *dCsk* using different Gal4 drivers caused distinct phenotypes. *dCsk* knockdown in a whole tissue increased the size of the tissue due to hyperproliferation of affected cells. In another approach, the *patched* (*ptc*) gene promoter was used to direct expression to a stripe of a few rows of cells along the anterior/posterior (A/P) boundary in wing discs (Speicher et al. 1994). In contrast to the whole-tissue knockdown, *ptc-gal4*-driven *dCsk* knockdown promoted apoptosis of affected cells in wing discs, which was similar to *dCsk* clones (Vidal et al. 2006). Notably, mutant cells near the A/P boundary had dropped out of the epithelial monolayer and migrated basally toward the posterior compartment and away from the *ptc* expression region. Genetic screening for modifiers of the *dCsk*-induced migration phenotype identified *Drosophila* orthologs of E-cadherin, JNK, and MMP1, as well as actin-remodeling genes such as Rho1. Careful analyses of the Src overexpression and *dCSK* mutant phenotypes have led to a model that Src signaling is biphasic: lower levels of Src pathway activity, such as those seen in *dCsk* mutants, result in pro-growth and anti-apoptotic signals; in contrast, strong levels potently induce apoptosis (Vidal et al. 2007). Together these results indicate that SFKs coordinate growth and invasion by multiple signaling pathways, ultimately altering the transforming cells' signaling network impacting its interactions with neighboring cells and promoting EMT (Vidal et al. 2006; Rudrapatna et al. 2014). Subsequently, dCsk was shown to also act through the Hippo pathway and require Yki for its growth regulatory functions (Kwon et al. 2015).

The Oncogenic Cooperation Model Involving Activated Notch

The pleiotropic signaling molecule Notch is activated in several human cancers (Aster et al. 2017). Similar to Ras activation, Notch activation by mutation, or expression of its ligand Delta, can promote tissue hyperplasia alone, but it is unable to push the tissue to full neoplasia (Dominguez and Casares 2005; Ferres-Marco et al. 2006). Following initial reports that Notch activation could cooperate with loss of cell polarity via mutation of the *scrib* gene to form a neoplastic tumor, a few other cooperating mutations have been identified (Brumby and Richardson 2003). The first identified that loss of the chromatin-modifying Polycomb group genes *lola* and

pipsqueak synergistically enhanced the overgrowth of Delta expressing tissues and triggered tumor dissemination partly through silencing of *Rbf* expression (Ferres-Marco et al. 2006). Strikingly, these tumors disseminate despite expression of differentiation markers, and further overexpression of the pro-neural differentiation factor Atonal could suppress the development of these tumors (Bossuyt et al. 2009). These results are similar to a cooperative model of Delta co-overexpression with Akt1 (Palomero et al. 2007). Large-scale chemical screening for suppression of tumor development in the Delta-Akt1 model led to the identification of NOS and LOX inhibitors which were able to selectively kill human leukemia cells (Villegas et al. 2018).

Notch activation also cooperates with the JNK/Stat target chinmo in oncogenesis, as well as fruitless to stimulate the formation of noninvasive tumors which strongly overgrow, fail to differentiate, and disrupt normal larval pupation (Doggett et al. 2015). This work supports a model that oncogenic BTB-Zn finger transcription factors including abrupt, chinmo, and fruitless contribute to invasive tumor growth through their ability to block the onset of differentiation.

Modifier screening by the group of Artavanis-Tsakonas has further identified multiple genes which enhance the Notch activation (N^{act}) phenotype, including multiple regulators of the cell cycle, chromatin modifiers, and regulators of the cytoskeleton (Ho et al. 2015). Their characterization of Mef2 or Src cooperating with N^{act} during neoplastic tumor development revealed that, similar to many other cooperative models, both tumors are dependent upon JNK pathway activation for their development (Ho et al. 2015; Pallavi et al. 2011). Intriguingly, however, they report that the activation of JNK in N^{act} + Mef2 tumors relies on the ligand Eiger, while the Src-dependent models appear to rely on cell intrinsic mechanisms of JNK activation, including ROS. These results could have important implications for the outcome of treatments which seek to inhibit TNF activation, the orthologous pathway in humans.

Modeling Human Cancers in Drosophila Eye Discs

RET Oncogene Activation to Model MEN2-Medullary Thyroid Cancer (MTC)

The inherited cancer syndromes of multiple endocrine neoplasia types 2A and 2B (MEN2A and MEN2B) and familial medullary thyroid cancer (FMTC) have each been associated with gain-of-function mutations of the Ret receptor tyrosine kinase (Leboulleux et al. 2004). Affected patients develop tumors of the neuroendocrine system affecting primarily the thyroid, parathyroid, and adrenal glands (Easton et al. 2000; O'Riordain et al. 1995; Ponder 1999). Due to developmental roles of the Ret kinase, affected patients also commonly display abnormalities of the skin, skeletal system, and peripheral nervous system. While MEN2 exists in a familial form,

many patients are afflicted by cancer as a result of de novo mutations, precluding diagnostic screening (Carlson et al. 1994; Shirahama et al. 1998; van Heurn et al. 1999). The variability in age of onset and tumor location, coupled with the tendency of these tumors to be metastatic and therapy resistant, presents significant challenges in patient treatment (Quayle and Moley 2005). The Ret protein is highly conserved between humans and *Drosophila*, and Ross Cagan's group was able to engineer two forms of Ret which they expressed ectopically in the developing *Drosophila* eye to investigate the cell biology underlying Ret-dependent oncogenesis in vivo (Read et al. 2005). These two forms mimic the causal mutations most commonly identified in patients with MEN2A or MEN2B, respectively. They initially utilized this model to screen in vivo for genetic modifiers that could alter the transformative capability of these Ret forms (Read et al. 2005). They were able to identify that components of the Ras, Src, and JNK pathways as well as specific chromatin remodeling factors could modify the phenotype (Read et al. 2005). Furthermore, orthologs of these genes were identified as high-frequency targets of LOH in human patients with aggressive disease. Follow-up studies on the chromatin remodeling factor, Sin3A, identified that it is required for the expression of several regulators of the actin cytoskeleton, as well as dCSK, a major inhibitor of Src activity (Das and Cagan 2013). Thus, the net result of Sin3A loss is increased activation of Src and actin polymerization, stimulating the JNK pathway to promote metastasis and growth, explaining the link between the different classes of modifiers identified in their previous screen (Das and Cagan 2013). Chemical screens on the MEN2 model in *Drosophila* eyes led to successful identification of drugs that showed promising effects on inhibiting cancer growth and progression. These efforts have recently led to clinical trials for MEN2 patients, showing that *Drosophila* can be a system of choice to study not only the biochemical signaling and cellular interactions during tumorigenesis but also for "proof-of-concept" studies for identifying small molecule inhibitors/anticancer drugs.

Glioma Model: Glial Cell Migration into the Optic Stalk/Nerve

Gliomas are the most common tumors of the central nervous system. Especially glioblastoma (GBM) is rapidly fatal, with median survival of patients being less than 1 year (Stupp et al. 2005). In most cases GBM is hard to cure despite surgery, intensive chemotherapy, and radiotherapy. To establish effective therapeutics, significant effort has been focused on determining the mechanisms of GBM formation. The most frequent genetic alterations include activation of EGFR (epidermal growth factor receptor) and PI3K (phosphatidylinositol-3 kinase) signaling pathways (Maher et al. 2001). To test the effects of these abnormalities in GBM development, Read et al. expressed activated isoforms of dEgfr and dp110 transgenes specifically in the glia in developing larval brain. Coactivation of these two pathways led to glial neoplasia (Read et al. 2009). Activation of either pathway alone showed milder or no effects, indicating that their concurrent activation was necessary for GBM

formation. The authors also found that the neoplastic transformation required multiple pathways dysregulated in human GBM, including cyclins-Cdks and RB-E2F, suggesting new therapeutic strategies for slowing GBM progression. In parallel, another model of glioma was developed in the eye discs by manipulating gene expression in glial cells that are known to migrate from the brain to the eye disc during development (Witte et al. 2009). In the eye discs, glial overexpression of activated epidermal growth factor receptor (EGFR) or the downstream kinase PI3K resulted in enhanced proliferation and migration of larval glial cells resulting in diffuse tumor-like enlargement of the optic stalk (Witte et al. 2009). Ectopic invasion of glial cells along the optic nerve was also observed. The effects of other key pathways on glial growth were also reported, for example, overexpression of activated *pvr* (a platelet-derived growth factor receptor/vascular endothelial growth factor receptor homolog in *Drosophila*) led to migration of glial cells along the optic nerve, whereas expression of activated *heartless* (htl, a fibroblast growth factor receptor 1 homolog in *Drosophila*) and INR (insulin receptor) showed markedly elevated numbers of glial cells in the optic stalk. Recently, we developed a glioma model in flies where the PI3K and oncogenic Ras pathways are coactivated in *Drosophila* glial cells in the developing larval brain and show that Yki activity is high in these tumors (Minata et al. 2019; Waghmare et al. 2014). The *Drosophila* models have also come in handy to test evolutionarily conserved regulatory relationships, e.g., the transcriptional control of ALDH1A1 by the FOXO-D transcription factor in flies and humans (Waghmare et al. 2014; Cheng et al. 2016) or the modification of Yki/YAP-mediated glioma growth by Tep1/CD109 (Minata et al. 2019). Overall, the *Drosophila* cephalic complex has generated important insights on glial neoplasms.

Tuberous Sclerosis Complex Model

Drosophila eye discs were informative in defining the developmental roles and signaling relationships of other disease-linked tumor suppressor genes. For example, tuberous sclerosis complex (TSC) is a human syndrome caused by mutations in TSC1 or TSC2 tumor suppressor genes and defined by widespread benign tumors. The mutations were known for a long time; however, the underlying molecular mechanisms that play a causal role remained unknown (Pan et al. 2004). Studies in *Drosophila* (later confirmed in mammalian models) showed that the TSC1/2 complex functions as a GAP (GTPase-activating proteins) for Rheb (a Ras-like small GTPase) to regulate TOR (target of rapamycin) signaling in response to nutrient-stimulated growth (Zhang et al. 2003). Thus, mutation of Tsc1/2 results in *Drosophila* genetic screens in the eye discs not only resulted in identification of components of this signaling network but also defined the molecular mechanisms involved in TSC-TOR signaling pathway emphasizing that *Drosophila* studies are a powerful tool for understanding the molecular mechanisms of the activity of human disease genes.

Cancer Cachexia Model

Cachexia is a multifactorial wasting syndrome associated with chronic disorders including cancers. It is a type of energy balance disorder in which an imbalance emerges between energy intake and its increased consumption by the body (Aoyagi et al. 2015; Fearon et al. 2013). Cachectic cancer patients suffer from significant weight loss primarily due to loss of skeletal muscle and fat in the body. It occurs in 40–90% of cancer patients depending on cancer type, and it accounts for about 20% of all cancer deaths (Argiles et al. 2014). At present, there is no cure for patients suffering from cachexia nor a biomarker to identify patients at high risk of developing it. How cachexia emerges in cancer patients has not been thoroughly determined yet, but *Drosophila* models for this disorder are emerging and generating what appear to be clinically relevant results. In 2015, two groups using *Drosophila* identified an insulin signaling antagonist, Impl2, as a tumor-derived factor driving cachexic phenotypes in two different *Drosophila* models (Figueroa-Clarevega and Bilder 2015; Kwon et al. 2015). Of note, hemolymph sugar levels were higher in flies with resident tumors, suggesting the emergence of insulin resistance; this mirrors the insulin resistant that frequently emerges in cancer patients. Because the mammalian ortholog IGFBP is known to antagonize insulin/IGF signaling (Baxter et al. 2014), this study suggests that proper control of insulin/IGF signaling is required to prevent wasting symptoms. Furthermore, Huang et al. showed that the related molecule IGF3B can induce skeletal muscle wasting in mice and is highly expressed in pancreatic ductal adenocarcinoma patients (PDAC). Of note, an estimated 90% of PDAC patients suffer from cachexia, and aggressive PDAC tumors are known to feature high degrees of YAP activation (Huang et al. 2016). Strikingly, both of the fly tumor models inducing cachexia depended upon activation of Yorkie, the homolog of YAP. These studies support a model that impaired insulin/IGF signaling contributes to cachexia development. It will be intriguing to ascertain if IGFBP can be a useful therapeutic target for this devastating syndrome. Importantly, these studies indicate that screening in *Drosophila* can identify relevant molecules affecting this process.

Drosophila and the Hallmarks of Cancer

The hallmarks of cancer include escape from cell death and senescence signals, the acquisition of unlimited growth/replicative potential, invasive behavior and EMT promoting metastases, altered cellular metabolism, genome instability and mutation, vascular recruitment or vasculogenesis, and interactions with the immune system to escape surveillance or to promote growth in response to inflammatory signals (Hanahan and Weinberg 2011). The vast majority of these have been successfully modeled in the *Drosophila* system, and the results have yielded molecular insights into how altered signaling and gene expression contribute to these processes

(Tipping and Perrimon 2014; Christofi and Apidianakis 2013; Mirzoyan et al. 2019). Throughout the chapter we have discussed results obtained in the *Drosophila* EAD relevant to many of these hallmarks, including unlimited replicative potential, escape from cell death and senescence signals, and metastases. Here we will discuss some other recent insights into the remaining and emerging hallmarks of cancer including genomic instability, vasculogenesis, altered metabolism, and immune/inflammatory interactions.

Chromosomal Instability

Chromosomal instability (CIN) is one of the hallmarks of cancer which generates genetic variation by altering the chromosome number (loss or gain of whole chromosomes known as aneuploidy) or structure (loss or gain of functions of chromosomes). It is proposed to help cancer cells to adapt to stressful environments and promote drug resistance (Tanaka and Hirota 2016). Downregulating spindle assembly checkpoint genes *bub3* and *rod* in *Drosophila* eye and wing imaginal discs produces aneuploid cells due to segregation errors, resulting in chromosomal instability (Dekanty et al. 2012). However, these imaginal discs also exhibit ectopic JNK and MMP1 expression leading to extrusion and apoptosis of these aneuploid cells. Blocking cell death of these cells by expressing baculovirus protein P35 resulted in strong overgrowth in the aneuploid cell population. When implanted in the abdomen of adult female fly, these P35 expressing aneuploid cells copiously overgrew highlighting their tumorigenic potential. Based on these observations, downregulating *bub3* or *rod* genes along with expression of *P35* in the *Drosophila* eye and wing imaginal discs was further used as a model to understand the molecular mechanism of chromosomal instability-induced tumorigenesis (Muzzopappa et al. 2017a; Clemente-Ruiz et al. 2016; Benhra et al. 2018). In yet another model, cooperation between microRNA, miR-8, and EGFR resulted in tumorigenesis. Peanut, a septin family protein, is an essential miR-8 target gene. Downregulation of peanut by miR-8 in the wing imaginal disc of *Drosophila* results in cytokinesis failure and the production of polyploid cells. Further studies showed cytokinesis failure due to $peanut^{RNAi}$ resulted in JNK activation, DIAP1 downregulation, and apoptosis. During tumorigenesis, *peanut* RNA can cooperate with EGFR overexpression to induce neoplastic transformation (Eichenlaub et al. 2016). Similarly, Yorkie overexpression during cytokinesis failure regulates the cell cycle phosphatases and overcomes JNK pathway-mediated tumor suppression leading to tumorigenesis (Gerlach et al. 2018). Together these results have begun to shed light on the cellular pathways that are affected by aneuploidy and how they can contribute to tumor initiation and progression.

Modeling Angiogenesis

As tumors grow they characteristically display areas of hypoxia which present a challenge to cell viability and further growth. An emerging hallmark of cancer encompasses the strategies deployed by tumor cells to overcome this challenge, both through de novo angiogenesis and via co-option or remodeling of the host's system. Strategies include vascular co-option (VC), intussusceptive angiogenesis (IA), and vascular mimicry (VM) (Donnem et al. 2013). VC most typically occurs with tumors developing in highly vascularized tissues in which tumors attach to and grow around nearby blood vessels, and disseminating tumor cells are found to migrate along these vessels. IA is a process that causes nearby vessels to split to form microcapillaries and is more rapid than VC. VM encompasses processes in which tumor cells can create their own tubelike networks to deliver oxygen and nutrients, or can integrate into vasculature among host endothelial cells, possibly through a transdifferentiation process. In vivo models for these processes remain limited, and the limited success of targeted therapeutic interventions indicates that we need a better understanding of the biology driving these processes. *Drosophila* have an open circulatory system and were thus thought to be a poor model for these processes. However, branched vascular structures referred to as trachea are used to locally deliver oxygen to tissues, and their development, branching, and remodeling are governed by similar molecular processes observed in mammalian angiogenesis (Zacchigna et al. 2008). Recently, it has been demonstrated that, as in human tumors, *Drosophila* imaginal disc tumors show high levels of hypoxic stress and nuclear localization of the HIF1α ortholog Similar (Sima) (Grifoni et al. 2015). The analysis performed in that work also provided evidence that (1) vascular co-option type behaviors and (2) vascular mimicry-type behaviors are clearly detected in *Drosophila* tumors. Finally, they provide evidence that the process of vascular mimicry relies on a JNK-dependent program in which cells reacquire normal levels of Polycomb expression and Stat activation and induce molecular markers of trachea differentiation. Thus, while this work focused on tumor development in the wing imaginal disc, it establishes paradigm for studying this process in *Drosophila* and may provide the basis for future work that can improve our understanding of the mechanisms that regulate these processes as well as if the mutational status of the tumor impacts which strategies are utilized.

Alterations in Tumor Metabolism

Tumor cells display altered cellular metabolism. Decades ago, Otto Warburg described that the fundamental process of glucose metabolism was altered in cancer cells (Warburg 1956). His name has been given to this switch, known as the Warburg effect, in which pyruvate is preferentially converted to lactate, rather than Acetyl-CoA due to increased production of lactose dehydrogenase (LDH). As a result, cells

rely on glycolysis, rather than the TCA cycle and oxidative phosphorylation for energy production. In *Drosophila* wing imaginal disc tumors induced by the constitutive activation of the VEGF receptor Pvr, the sole LDH ortholog, *ImpL3*, becomes upregulated and highly active (Wang et al. 2015). As in the mammalian system, this is dependent upon stabilization by HIF1α (Sima), but they were able to demonstrate that this did not necessarily rely upon hypoxic conditions but rather on coactivation of the Ras and PI3K pathways. In parallel to the upregulation of LDH, pyruvate dehydrogenase kinase enzyme (PDHK), which is required to convert pyruvate to Acetyl-CoA to feed into the TCA cycle, is inactivated. Concomitantly, glycolytic enzymes are increased in expression, and electron transport chain components are diminished. One of the key regulatory interactions identified in this process was JNK-dependent activation of the enzyme PDHK, which in turn inactivates pyruvate dehydrogenase. In a separate neoplastic tumor model driven by EGFR overexpression coupled with loss of *pipsqueak*, *Impl3/LDH* was likewise upregulated along with upregulation of glucose transporters and increased glycolytic flux (Eichenlaub et al. 2018). The authors were further able to demonstrate that LDH could cooperate directly with EGFR overexpression to promote neoplasia. Glucose transporter upregulation was also shown to be required for tumor growth, and it was further demonstrated that a high-sugar diet could promote neoplasia in discs which overexpress EGFR alone.

A high-sugar diet can contribute to obesity. Obesity is a rising public health issue and is increasingly also correlated with increased cancer risk (Giovannucci et al. 2010). A *Drosophila* model for assessing the interplay between obesity and type 2 diabetes has been established in which high levels of dietary sugar result in larval obesity, high levels of circulating insulin, and emerging insulin resistance in normal tissues, mimicking important clinical features of insulin resistance in type 2 diabetes (Musselman et al. 2011). Of note, while Ras + Src-activated MARCM clones in the eye on a normal diet are normally eliminated by apoptosis, tumor initiation on the high-sugar diet resulted in aggressive growth and secondary tumor formation (Hirabayashi et al. 2013). In contrast to the normal tissues, the tumors remained sensitive to insulin and even upregulated expression of the insulin receptor. Insulin signaling within the cells leads to Yki activation (Hirabayashi and Cagan 2015). The growth and metastasis synergy in Ras + Src depended upon JNK- and Yorkie-dependent upregulation of wingless expression (Hirabayashi et al. 2013; Hirabayashi and Cagan 2015). Interestingly, Ras+scrib tumors did not increase their growth and did not disseminate secondary tumors in response to high-sugar diets, indicating that tumor genotype may play a role in this response.

Finally, tumor metabolic changes are not solely restricted to changes in glucose metabolism but also affect other pathways including amino acid flux. Similar to an emerging feature of human tumors, *Drosophila* tumors can be glutamine dependent (Willoughby et al. 2013). This may be a conserved process from flies to man and can be triggered, in part, by inactivation or loss of Rb, a common feature in many tumors (Nicolay et al. 2015). These results support the idea that the *Drosophila* imaginal discs can play an important role in deciphering the changes in cellular

metabolism in tumors which may lead to identification of ways to target this difference between tumor and normal cells in therapies.

Escape from Immune Surveillance/Tumor-Promoting Inflammation

The interactions between the cells of the innate immune system, inflammatory signaling, and the tumor are historically complex. Compelling evidence supports that these processes can function as both a tumor suppressor mechanism and a tumor-promoting mechanism in vivo. Using the *Drosophila* eye imaginal disc tumor models, multiple groups have examined the interactions between cells of the innate immune system (hemocytes) and the tumor. Initial reports demonstrated that hemocytes specifically associate with ruptures in the basement membrane associated with invasive tumors (Pastor-Pareja et al. 2008). Further, they showed that the presence of the tumor stimulated hemocytes to proliferate in a JAK-STAT-dependent fashion. The association of hemocytes with the tumor initially seems to be a tumor suppressor mechanism, as ablation of the hemocytes leads to the growth of larger tumors. Further study has revealed that this initial tumor suppression occurs through secretion of the TNF ortholog Eiger by the hemocytes, stimulating apoptosis in the receiving tumor cells. However, in the presence of oncogenic Ras signaling, the tumor response to the signal shifts from apoptosis to cell proliferation and migration, thus revealing a mechanism for how innate immune cells can both suppress and promote tumor development (Cordero et al. 2010). In addition, Eiger produced by the tumor is able to stimulate signaling in the tumor-associated hemocytes which relay the signal to the fat body, activating Toll signaling there, provoking a systemic inflammatory response (Parisi et al. 2014). Thus, despite the lack of an adaptive immune system, *Drosophila* can yield important insights into the mechanisms that drive systemic responses to tumor development as well as into cross talk between the tumor and cells of the innate immune system.

Signaling Pathway Activation and Cross Talk in Tumor Development

Normal cells respond to hyperactivation of Ras signaling with mild increases in proliferation, increased apoptosis, and differentiation or senescence (Karim and Rubin 1998; Halfar et al. 2001). Notch activation has similar effects in normal cells (Ho and Artavanis-Tsakonas 2016). However, the cooperative oncogenesis models have revealed that, in cells with disrupted apicobasal polarity or altered cytoskeletons, these pathways can become primary drivers of tumorigenesis, facilitating growth, survival, and invasion (Brumby and Richardson 2003; Pagliarini and Xu

2003; Andersen et al. 2015; Brumby et al. 2011; Khoo et al. 2013). These altered cell responses have led many investigators to try to understand how signaling pathway activation, downstream effectors, and pathway cross talk are altered during tumor development. Among these we will focus on the recurrent emergence of JNK and Stat signal activation, coupled with the inactivation of the Hippo pathway (Yki activation). In this context we will discuss points of pathway cross regulation as well as synergistic and atypical target gene expression by these pathways.

Over the past decade, a major push in the *Drosophila* tumor research field has been to determine how cellular responses to Jun kinase (JNK) signaling are altered in tumors. In normal epithelial cells, ligand-dependent stimulation of JNK signaling triggers apoptosis (Andersen et al. 2015; Igaki et al. 2002; Kanda et al. 2002). In line with this behavior, JNK signaling functions as a tumor suppressor in subsets of tumors that arise from chromosomal instability/aneuploidy, endocytosis defects, and a subset of hyperplastic tumors (Bossuyt et al. 2009; Dekanty et al. 2012; Gerlach et al. 2018; Woodfield et al. 2013). However, this is in stark contrast to most neoplastic tumors which depend on JNK signaling for their growth and/or invasive capabilities (Chi et al. 2010; Das and Cagan 2013; Dekanty et al. 2012; Brumby et al. 2011; Igaki et al. 2006; Uhlirova and Bohmann 2006; Wu et al. 2010b; Jiang et al. 2011; Poon et al. 2018).

To date, however, the exact molecular switch that facilitates the switch of JNK signaling from tumor suppressor to tumor promoter remains unclear. A second TNF receptor, Grindelwald, was recently identified and implicated in mediating JNK activation in response to Eiger binding in cells that lack apicobasal polarity (Andersen et al. 2015). Alternatively, recent findings implicate alterations in the upstream regulation of JNK in invasive tumors away from TNF receptor-based inputs to cell intrinsic inputs, including ROS production, which may contribute to the alteration of JNK outputs (Ho et al. 2015; Muzzopappa et al. 2017b; Manent et al. 2017). Finally, a positive feedback loop between caspase-dependent ROS production and JNK has been identified wherein tumor-produced ROS stimulates tumor-associated hemocytes to produce Eiger which in turn maintains JNK activation (Perez et al. 2017; Cordero et al. 2010; Pastor-Pareja et al. 2008). Also, at the upstream level, EGFR activation can switch the activity of activated JNK from inhibiting to promoting the activity of Yki via promoting actin polymerization to support tumor growth (Enomoto et al. 2015). Further studies will be needed to determine which of these mechanisms are interlinked, or independent, and if they are a common feature of many tumor types, or tumor-type specific. Downstream of JNK activation, AP-1 type transcription factors other than Jun and Fos, including ATF3, Pdp1, and IRBP18 (CG6272), have been identified as being highly induced in multiple tumor types (Kulshammer et al. 2015; Atkins et al. 2016; Pascual et al. 2017; Donohoe et al. 2018). Interestingly, the induction of Pdp1 and ATF3 seems to lie downstream of Yki activation, yielding another point of cross talk between the two pathways (Pascual et al. 2017). The identification of these transcription factors as being induced, which is important for the full manifestation of tumor phenotypes, introduces the possibility of novel target gene induction in response to JNK signaling. However, their exact roles in tumorigenesis have not been fully assessed; recent

findings do suggest that ATF-3 may play an important role in multiple phenotypes triggered by apicobasal polarity loss, which also activates JNK signaling (Donohoe et al. 2018). Downstream, an expanding panel of validated AP-1 target genes in tumors is also shedding light on how JNK signaling can promote tumor cell invasion, as well as proliferation and survival. Mmp1 and the actin cross-linker *cheerio* are induced and support invasive behavior (Uhlirova and Bohmann 2006; Kulshammer and Uhlirova 2013). Cheerio also is required for the full growth potential of the tumor. Additional validated growth-promoting targets include the wound-responsive enhancer of *wingless* expression, *chinmo*, the potential Ras effector *Ets21C*, and the *unpaired* genes 1–3 which are ligands of the Stat pathway (Doggett et al. 2015; Ho et al. 2015; Dekanty et al. 2012; Kulshammer et al. 2015; Bunker et al. 2015; Toggweiler et al. 2016; Zhang et al. 2019).

More recently, an increased amount of attention has also been applied to the activation of Stat signaling and inhibition of the Hippo pathway to promote tumor growth (Classen et al. 2009; Gonzalez et al. 2009; Doggett et al. 2011). Similar to the activation of JNK, the activation of Stat and inhibition of Hippo/activation of Yki seem to be emergent properties of some tumors, as the pathways themselves are not direct targets of mutation in most of the models. A key discovery to explaining this phenomenon was that excess polymerization of F-actin, a common feature of many tumors, can trigger Yki activation (Sansores-Garcia et al. 2011; Fernandez et al. 2011). As previously mentioned, Yki activation is observed to promote tissue growth and cell survival, with known targets *Myc*, *Cyclin E*, *Diap-1*, and *string* (Huang et al. 2005; Wu et al. 2003; Gerlach et al. 2018; Ziosi et al. 2010; Neto-Silva et al. 2010). However, in the tumor context, Yki activation can maintain JNK activation, further stabilizing the tumor signaling environment (Ma et al. 2015). In addition to its aforementioned cooperation with AP-1 transcription factors to increase the expression of the Stat pathway ligands *upd1*, *upd*2, and *upd3*, there is also evidence that the Yki target miRNA *bantam* directly inhibits accumulation of the Stat pathway feedback inhibitor SOCS36E, providing a mechanism to prevent Stat pathway inactivation when Yki is activated during tumorigenesis (Herranz et al. 2012). Stat pathway activation is in turn a key driver of tumor growth and invasion (Ho et al. 2015; Wu et al. 2010b; Classen et al. 2009; Gonzalez et al. 2009; Ekas et al. 2010; Flaherty et al. 2010; Torres et al. 2018; Davie et al. 2015).

Other tumor models involving genes that can cooperate with Ras activation to generate neoplastic growth may offer further insight into how signaling pathways and cross talks are rewired during tumorigenesis. In a tumor model of cooperation of Hippo pathway impairment (*wts*$^{-}$) with Ras activation, a global transcriptome analysis has provided insight into how the differentiation function of Ras signaling is reprogrammed to promote tumorigenesis, by showing that Yki elevates the expression of the Ras target gene, *pointed*, which is crucial for the synergistic tissue growth (Pascual et al. 2017; Bosch et al. 2016). Independently, it was shown in the eye-antennal epithelial tissue that Ras^{V12} can cooperate with loss of lysosomal gene function to cause neoplastic overgrowth, potentially dependent on ROS accumulation (Chi et al. 2010; Manent et al. 2017). Additionally, mutations in the chromatin remodeling gene, *polyhomeotic (ph)*, cooperate with Ras^{V12} in a clonal context to

induce eye-antennal tissue neoplastic tumors, which depends on Notch pathway activation (Martinez et al. 2009). However, loss of *ph* and other Polycomb complex genes alone, when generated in a whole eye-antennal epithelial tissue, can also result in neoplastic tumors, which in this context is dependent on ectopic Upd-JAK-STAT signaling (Classen et al. 2009; Martinez et al. 2009; Beira et al. 2018). These differences may depend on the level of expression and the region of the tissue affected, but, additionally, in the clonal context, the induction of cell competition might affect the cooperative mechanism involved in neoplastic tumor formation. Together these models have helped to illuminate how specific mutations result in the aberrant activation or rewiring of numerous signaling pathways in tumors.

Regulation of Tumor Gene Expression

To date, several groups have looked at transcriptome-wide changes in tumor gene expression and the open chromatin landscape. From studies focused on differential expression of genes in cooperative models, it has become clear that, as is observed in the tumor phenotype, gene expression changes are not merely additive. Rather, they contain synergistically activated or repressed genes (Kulshammer et al. 2015; Atkins et al. 2016; Pascual et al. 2017; Turkel et al. 2013). Each of these studies identified common differentially upregulated transcription factors including AP-1 type (Kayak, ATF-3, Pdp-1), bHLH (Myc and Taiman (SRC3)), nuclear hormone receptor (Ftz-f1), and Ets21C, along with activated Stat. Together, these studies provide evidence that these factors cooperate in orderly transcription factor networks to drive the expression of tumor-specific genes and that each is important for tumor development. Of note, transcriptomes of single-mutation neoplastic discs also showed upregulation of most of these same factors (Bunker et al. 2015). A brief comparison of these aforementioned datasets, however, indicates that Ets21C may be more relevant in tumors where the canonical ETS transcription factor pointed is expressed at lower levels (Kulshammer et al. 2015; Atkins et al. 2016) and that Taiman may play a larger role in tumors that depend on loss of *scribble* rather than other forms of polarity loss (Atkins et al. 2016; Turkel et al. 2013; Zhang et al. 2015). In addition to changes in gene expression, analysis of the chromatin landscape of Ras^{v12}; $scrib^{-/-}$ tumors found that it is altered from the normal state and that a distinct landscape of cis-regulatory transcription factor binding sites are available in tumor cells. Interestingly, the key regulators of the more accessible regions in tumor cells were identified as AP-1, Stat, Zelda, and Sd (the transcription factor partner of Yki) (Davie et al. 2015). This altered chromatin landscape may be an important key to understanding how target gene expression is affected. For example, canonical Notch targets of the E(spl)cluster are largely not induced in Notch-dependent tumors, while Su(H) remains responsive (Ho et al. 2015). Similarly, an analysis of differential expression of predicted targets of Sd in $Ras^{v12}scrib^{RNAi}$ tumors reveals that a subset of targets is strongly upregulated in the tumors, but a distinct subset is strongly downregulated, when compared to control discs (Atkins et al.

2016). It remains to be determined if shifts in chromatin accessibility, or the absence of necessary co-regulators, are the primary drivers of these observations.

Finally, recent insights into cooperative regulation of AP-1-, Stat-, and Yki-dependent target genes are revealing molecular mechanisms behind the observed synergistic gene expression profiles. For example, the AP-1 target gene Ets21c encodes a transcription factor that is indicated in the cooperative regulation of the AP-1 targets *mmp1*, *Pvf1*, and *upd1* (Toggweiler et al. 2016). Furthermore, induction of *upd3* expression in tumors depends upon cooperation between Yki and AP-1 for full activation (Bunker et al. 2015). Of note, multiple in vitro studies using human cancer cells have identified co-regulation of tumor-specific targets by the Yki/Sd human orthologs YAP/TEAD with AP-1 transcription factors (Verfaillie et al. 2015; Liu et al. 2016; Zanconato et al. 2015). Similarly, both AP-1 and Stat are known regulators of *chinmo* expression, though it is not clear if they are each sufficient or if they cooperate in their regulation (Doggett et al. 2015; Flaherty et al. 2010). Furthermore, both Yki/Sd and Stat have also been independently shown to promote *cdc25* (a.k.a. *string*) expression in tumors, but again it remains undetermined if their regulation is cooperative (Gerlach et al. 2018; Davie et al. 2015). Cumulatively, these results paint an emerging picture of tumor development as a distinct cellular fate that occurs as the result of synergy beyond the additive effects of the initiating mutations.

Concluding Remarks

Here we have presented a cross section of the knowledge and insights we have gained from using the *Drosophila* EAD as a model system to understand the genetic and cellular changes that occur during oncogenesis. From the isolation and maintenance of the first spontaneous tumor suppressor mutant in the 1930s, until modern explorations of transcriptomics and chromatin landscapes, this model system has consistently shed light on the processes that drive tumor development and cancer progression. In recent years *Drosophila* is also emerging as an important tool for the identification of clinically relevant drugs that show reduced cytotoxicity, a critical bottleneck in drug discovery (Das and Cagan 2013; Parsons et al. 2017). It is also an emerging system for the analysis of processes that affect patients beyond the tumor, including interactions with the immune system and cachexic wasting. With continued tool development and innovation, it is nearly certain that this model system will continue to yield critical insights into cancer biology.

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Recent Contributions of the *Drosophila* Eye to Unraveling the Basis of Neurodegeneration

Pedro Fernandez-Funez and Ryan R. Myers

Abbreviations

AD	Alzheimer's disease
ALS	Amyotrophic lateral sclerosis
Atx	Ataxin
Aβ	Amyloid-β peptide
C9orf72	*Chromosome 9 open reading frame 72*
CJD	Creutzfeldt-Jakob disease
DPR	Dipeptide repeats
ERG	Electroretinogram
FTD	Frontotemporal dementia
GFP	Green fluorescent protein
GMR	Glass multiple reporter
GOF	Gain-of-function
GSS	Gerstmann-Sträussler-Scheinker
Hsp70	Heat shock protein 70
LOF	Loss-of-function
PrP	Prion protein
RAN translation	Repeat-associated non-ATG translation
SCA	Spinocerebellar ataxia
TDP-43	TAR DNA-binding protein 43
TEM	Transmission electron microscope
WT	Wild type
α-syn	α-Synuclein

P. Fernandez-Funez (✉) · R. R. Myers
Department of Biomedical Sciences, University of Minnesota School of Medicine, Duluth, FL, USA
e-mail: pfernand@d.umn.edu

A. Singh, M. Kango-Singh (eds.), *Molecular Genetics of Axial Patterning, Growth and Disease in Drosophila Eye*,
https://doi.org/10.1007/978-3-030-42246-2_10

Using the *Drosophila* Eye to Understand Human Neurodegenerative Diseases

Drosophila is a small fly highly recognized for its easy laboratory manipulation and genetic prowess. More than 100 years ago, the Nobel Prize awardee Thomas H. Morgan adopted fruit flies for his laboratory work because of its 10-day life cycle coupled with easy maintenance. Another attractive feature of fruit flies as an experimental model is their relative complexity, including compound red eyes, clear wings with simple veins, invariable pattern of macrochaetae (large bristles), and polytene chromosomes, large enough to identify chromosomal rearrangements under the microscope. The work of Morgan's disciples led to the generation of hundreds of novel mutations and chromosomal rearrangements, making *Drosophila* one of the favorite organisms for genetic studies (Bellen et al. 2010). Successive generations of *Drosophila* enthusiasts have resulted in tens of thousands of mutations, and the integration of gene networks that later showed to be conserved in other organisms, including humans. *Drosophila* research is highly dynamic and rapidly adopts developing technologies, like genome sequencing completed in the year 2000 (Adams et al. 2000) and transgenesis in 1982 (Rubin and Spradling 1982). In many instances, *Drosophila* researchers have developed new technologies that continue to revamp its value as a research tool, like balancer chromosomes, mosaic analysis (reviewed in (Blair 2003)), and the UAS/GAL4 expression system (Brand and Perrimon 1993), to name a few. However, the biggest capital of *Drosophila* is its generous community that generates publicly available resources, including large collections of loss-of-function (LOF) and overexpression alleles, molecularly mapped insertions, tagged genes, websites like FlyBase (flybase.org), and other technologies for cell tracing and mosaic analysis (Nagarkar-Jaiswal et al. 2015; Yamamoto et al. 2014; Gratz et al. 2014; Bassett et al. 2013; Dietzl et al. 2007; Matthews et al. 2005).

In the first version of this book in 2013, we introduced the advantages of using the *Drosophila* eye to model neurodegenerative diseases and to unravel the cellular mechanisms mediating them. The *Drosophila* eye is composed of 600–800 ommatidia, the visual units of insects. The precise arrangement of hundreds of ommatidia creates a perfect lattice; slight changes to this lattice are easy to observe under the stereoscope without dissections, staining, or time-consuming manipulations. In addition, alterations in normal eye development can result in several phenotypes affecting the size, pigmentation, and/or organization of the eye. This easy access and richness of phenotypes provide the ideal conditions for fast evaluation of the impact of genetic manipulations in the eye. Among the best applications of the eye include highly risky projects that require a fast determination on the effect of a series of constructs and genetic screens in which thousands of constructs are tested.

Here, we provide a review of recent progress since the publication of the first version of this book. Although just 6 years have elapsed since the first edition, it is sobering to realize how novel applications continue to highlight the important contributions of the *Drosophila* eye to the recent progress of the field. We will focus this review not on all the work done with the traditional approaches but substantial

technical or conceptual advances that continue to illustrate the importance of this specific assay and its continued potential for future advances.

Technical Notes: Maximizing the Utility of Model Systems

For this chapter, we will emphasize the advantages of relatively recent technologies: ϕ31-dependent integration (precise attB/attP integration), the Q-system, and codon optimization. Since the generation of the first transgenic flies (Rubin and Spradling 1982), transgenes have been randomly inserted by transposase-mediated integration of engineered P-elements. However, these insertions are biased toward the 5'UTR of certain genes (hot spots) and, thus, can damage target genes and disrupt regulatory sequences. Additionally, P-elements can place constructs under the control of unknown regulatory domains (enhancer trapping), which complicates the direct comparison of a series of related constructs (WT vs mutant, isoforms, etc.) due to chromosomal microenvironment resulting in different expression levels (a.k.a., position effects). This problem can be neutralized by generating large sets of random insertions (10–20), followed by comparison of protein expression by western blot or mRNA levels by quantitative PCR. These are time-consuming approaches that would also mask differences in half-life and other posttranslational effects. The "bacterial" and "phage" attachment sites (attB/attP), which are recognized by ϕ31 integrase, were introduced in fruit flies with the purpose of generating clean insertions with defined molecular precision that could be reused for multiple constructs to provide the desired homogenous background (Bischof et al. 2007). Several well-characterized landing sites are currently available in different chromosomes. We have used the attP2 site on chromosome 3 in two published projects because it produces high expression (Moore et al. 2018; Fernandez-Funez et al. 2016).

A second technical development is the relatively new heterologous Q-system adopted from *Neurospora* (Riabinina et al. 2015; Potter et al. 2010). The Q-system is a binary system that works conceptually similar to the UAS/GAL4 but uses independent binding promoter sites (QUAS) and transcriptional activator (QF). The system also has a repressor (QS) that is inactivated by quinic acid. The Q-system was introduced for complex genetic experiments in the brain, including tracing neuronal lineages (Riabinina et al. 2015; Potter et al. 2010). Its main limitation is that it requires the generation of new tools like unique QF driver strains for specific spatial control and QUAS responder lines. However, this system is absolutely critical for performing highly complex manipulations that require two independent expression systems.

The third technical change we review here is codon optimization of heterologous genes. Expression of most amyloids connected with human proteinopathies cause robust and distinct eye phenotypes in *Drosophila* (Fernandez-Funez et al. 2013; Rincon-Limas et al. 2012). But there are two notable exceptions: α-synuclein (α-syn) and the prion protein (PrP), the amyloids associated with Parkinson's and prion disease, respectively. The lack of robust phenotypes limited the work with

these models to time-intensive experiments requiring the dissection of aged adult flies. Codon optimization can maximize translation of products whose expression is low or are targeted for degradation upon translation. Codon usage is typically not a problem when introducing foreign genes since sufficient protein is expressed to cause toxicity, even in the eye. Codon optimization can boost translation enough to bypass quality control mechanisms that target newly translated proteins for degradation. In the PrP case, this strategy led to new eye phenotypes that expanded the scope of experiments that could be conducted. The main problem is that full optimization requires large numbers of triplet changes, which precludes directed mutagenesis approaches. PrP is a small protein of 250 amino acids (210 for the mature protein), and its gene can be easily and economically synthesized, making codon optimization a minor extra step. Larger genes pose a more serious problem because synthesis of the entire gene is expensive and time-consuming. Synthesis of G-blocks can help with the synthesis of large genes, but it is a more significant step than with smaller genes.

The New Genetics of ALS/FTD: *Drosophila* Leads the Charge

Amyotrophic lateral sclerosis (ALS) is a motor neuron degenerative disease with complex genetics and pathology. The most common pathology in ALS is the accumulation of TDP-43 (TAR DNA-binding protein 43) aggregates in the cytosol, although SOD1 (superoxide dismutase 1), FUS (fused in sarcoma), and ubiquitin are also present in other cases. We already described genetic models of ALS expressing the mutant alleles for these three genes in the first version of this book (Fernandez-Funez et al. 2013). In 2011, *C9orf72* (*chromosome 9 open reading frame 72*) was identified as the most common gene mutated in familial ALS and familial frontotemporal dementia (FTD) (DeJesus-Hernandez et al. 2011; Renton et al. 2011). This discovery profoundly changed our understanding of these two diseases. First, the ALS/FTD connection was suspected for some time due to unexpectedly high rates of co-occurrence, but the mechanistic connection between motor neuron disease and dementia was missing. The discovery of the *C9orf72* link revealed a new entity—ALS/FTD—that had been hiding in plain sight for decades. Second, the mutations discovered in *C9orf72* were novel G4C2 hexarepeats ($GGGGCC_n$) in a noncoding 5′UTR. Other neurodegenerative diseases are caused by noncoding repeats (myotonic dystrophy 1 and 2; SCA10 and 12), but this is the first hexarepeat, which implies novel neurotoxic mechanisms.

Given the novelty of the $G4C2_n$ noncoding expansions, the challenge was to figure out *how* they cause the ALS/FTD pathology. The presence of $G4C2_n$ could alter transcription and translation, lowering the levels of the C9orf72 protein products. Additionally, mRNAs carrying noncoding $G4C2_n$ expansions have stable secondary and tertiary structures (hairpins and G-quadruplex). These mRNA structures are proposed to sequester nuclear proteins involved in splicing, resulting in missplicing of many targets (Scotti and Swanson 2015; Zhang and Ashizawa 2017).

This toxic mRNA mechanism has been proposed for other noncoding repeats, including myotonic dystrophy type 1 (MD1), MD2, SCA10, and SCA12, among others (Scotti and Swanson 2015; Zhang and Ashizawa 2017). However, these repeats can also be translated by an unusual RAN (repeat-associated non-ATG) translation mechanism (Zu et al. 2011). Some factors of the standard translational machinery are likely primed by tertiary mRNA structures leading to the initiation of translation in the absence of ATG. Moreover, RAN translation occurs *in the three reading frames and from both strands*!! In the case of *C9orf72*, the $G4C2_n$ hexarepeat can produce three different dipeptide repeats (DPR) from the sense strand (PG, GA, GR) and three from the antisense strand (GP, AP, PR), for a total of five different DPR (PG = GP) (Zu et al. 2013). These DPRs are a new phenomenon because RAN translation had been previously described for triplet repeats that generate homopolypeptides (e.g., polyQ, polyS, polyA) (Zu et al. 2011). RAN translation further complicates the identification of toxic mechanisms caused by noncoding repeats because of the three coexisting pathologies: LOF, mRNA toxicity, and RAN polypeptides. How can the contribution of each of these agents be isolated and tested independently in relevant assays? Moreover, how can the toxicity of DPRs be tested without the contribution of the repeat-containing mRNA and vice versa in light of RAN translation? Testing these ideas in an animal model would be desirable, but the number of constructs and the risk that they may not work introduced a considerable risk. *Drosophila* proved to be an ideal model for helping solve this enigma because of the efficient generation of transgenic animals and the ability to manipulate multiple genotypes in parallel.

The first animal model of *C9orf72*-related repeat expansions was done in the *Drosophila* eye and supported the hypothesis that noncoding $G4C2_n$ repeats were pathogenic (Xu et al. 2013). Answering the questions about the species responsible for toxicity required generating many constructs and developing ingenious solutions to the need of expressing noncoding $G4C2_n$ repeats without RAN products AND vice versa. First, to create noncoding $G4C2_n$ repeats that could not encode RAN peptides (termed *RNA-only*), the authors introduced multiple stop codons in both strands (Mizielinska et al. 2014). Due to the innate instability of expanded repeats, the investigators used a creative cloning method termed recursive directional ligation to generate strands of stable expanded repeats. Six-base pair sequences containing stop codons for every sense and antisense frame were inserted every 12 G4C2 repeats to prevent translation. Constructs were introduced in flies into attP landing sites, ensuring comparable expression levels. Expression of uninterrupted noncoding $G4C2_{36}$ showed mild toxicity in the eye, whereas noncoding $G4C2_{103}$ showed small, disorganized, depigmented eyes (Mizielinska et al. 2014). In contrast, neither RNA-only $G4C2_{108}$ nor RNA-only $G4C2_{288}$ showed eye phenotype. Adult survival (longevity) assays further supported the lack of toxicity of these RNA-only $G4C2_n$ constructs. These experiments suggested that the toxicity of noncoding $G4C2_n$ repeats required the expression of RAN DPRs. The only caveat to these studies is that the introduction of multiple stop codons in both strands interrupted the long $G4C2_n$ sequences required for the stability of tertiary mRNA structures, although they still accumulated in RNA foci in cultured cells and in *Drosophila*

polytene chromosomes. Furthermore, the authors confirmed that the insertion of stop codons did not affect the formation of the G-quadruplex structure characteristic of G4C2 repeat RNA.

The other challenging question was comparing the toxicity of the five different DPRs (Mizielinska et al. 2014). Are all of them toxic? Are they equally toxic? The idea was to generate constructs encoding each DPR by adding ATGs upstream of the $G4C2_n$ repeats in the appropriate reading frames. To avoid the formation of RNA foci and tertiary mRNA structures, the authors introduced *alternative codons* taking advantage of the degenerate genetic code that broke the continuous $G4C2_n$ repeats. This was confirmed in more sensitive longevity assays. GR36 and PR36 led to small and disorganized eyes, with GR36 resulting in stronger phenotypes. To determine if PA36 and GA36 could induce abnormal eyes with longer repeats, the authors generated constructs expressing PA100 and GA100. These longer constructs still showed no eye phenotypes, although GA100 showed mild toxicity in the survival assay. In contrast, GR100 and PR100 resulted in very small and disorganized eyes, highlighting the dramatic differences between the DPR containing arginine (GR, PR) vs those containing alanine (PA, GA). Overall, this study highlighted the strengths of *Drosophila* to efficiently create multiple constructs, while the eye assay provided a fast assay to compare the relative toxicity of each construct. This project was highly innovative but also carried extensive risk; thus, a fast and inexpensive organism seemed appropriate to answer these complex questions.

Shorter and Longer Amyloid-β Peptides

Alzheimer's disease (AD) is the most prevalent neurodegenerative disease and a growing concern in advanced and developing economies (Alzheimer's Association 2019; Lane et al. 2018). Several independent groups published *Drosophila* models of amyloid-β42 neurotoxicity (Aβ42) or tau neurotoxicity with particular use of eye assays (Finelli et al. 2004; Iijima et al. 2004; Crowther et al. 2005; Casas-Tinto et al. 2011; Jackson et al. 2002; Wittmann et al. 2001). The Aβ models were based on the expression of Aβ40 and/or Aβ42 and differed mainly on the robustness of the eye phenotype. We rationally designed our own model to express high levels of Aβ42 (two copies of Aβ42 per construct), which proved critical to complete a genetic screen and identify suppressors of Aβ42 neurotoxicity (Casas-Tinto et al. 2011). More recently, we faced the challenge of generating *Drosophila* models for seven different Aβ peptides physiologically produced by sequential cleavage by β- and γ-secretases. Under normal physiological conditions, high levels of Aβ40 accumulate in the brain possibly as a byproduct during the cleavage of the amyloid precursor protein (APP) into extracellular and nuclear fragments. Under pathological conditions (e.g., AD), the cleavage site of γ-secretase shifts slightly in the transmembrane domain of APP leading to the production of the slightly longer Aβ42. Aβ42 is prone to misfolding and the formation of highly toxic oligomers (Moore et al. 2018). Remarkably, several additional Aβ peptides can be detected in the

human brain tissue as normal physiological products of APP metabolism: Aβ36, Aβ37, Aβ38, Aβ39, and the longer Aβ43. These Aβ peptides are generated by slippage in the cleavage site of γ-secretase. Since these seven peptides accumulate naturally, the questions were as follows: Do they contribute to AD pathogenesis? Do they increase Aβ42 toxicity by promoting its aggregation, or are they protective by interfering with Aβ42 aggregation?

Drosophila seemed an ideally suited animal model to handle this many transgenes. To compare the relative toxicity of each Aβ construct, we inserted them in the attP2 site on chromosome 3 that we had used previously (Fernandez-Funez et al. 2016). Once we inserted all the Aβ constructs in attP2, we crossed them with the *GMR-Gal4* driver to compare their relative toxicity in the eye. As expected, Aβ42 showed a weakly disorganized eye when the crosses where raised at 28 °C to push Gal4 activity (Moore et al. 2018). The Aβ36 to Aβ40 peptides showed no phenotype, whereas Aβ43 showed a very weak disorganization.

To determine if higher expression levels of these peptides were toxic in the eye, we generated flies expressing two copies of the Aβ peptides with one copy of *GMR-Gal4*. Flies expressing 2× Aβ42 showed the same phenotype we described for flies carrying two tandem copies of Aβ42 (Casas-Tinto et al. 2011); 2× Aβ43 showed weak but clear eye disorganization (Moore et al. 2018). In contrast, the 2× Aβ36 to Aβ40 peptides showed no eye phenotype at all. Finally, we used the eyes to determine the consequence of combining short Aβ peptides with Aβ42. Interestingly, some Aβ combinations showed mild reduction of the Aβ42 eye phenotype, which we later confirmed in climbing assays. We would have not done these combination experiments in climbing assays without preliminary evidence that they modified Aβ42 neurotoxicity.

Overall, the lessons from this work were pretty clear. These experiments demonstrated the advantage of starting the experiments in the eye because they provided initial observations and hypotheses that we could later test in the behavioral assay. Our main concern about the attP2 landing site being weaker than strong random insertions was confirmed, but Aβ42 expression was sufficient to observe a weak eye phenotype that we could use as a reference. Creating flies expressing two copies of each Aβ construct took additional time, but this approach answered the questions about the lack of toxicity of the short Aβ peptides. One final note: in western blot, ELISA and immunofluorescence assays Aβ36 showed very low levels compared to other Aβ peptides. If we had tried to identify Aβ lines with similar expression levels by western blot, we would have invested time and resources trying to find Aβ36 lines comparable to the other Aβ peptides.

New and Improved PrP Models: Pulling All the Stops for a New Phenotype

For more than a decade, we have worked with several models of prion diseases in *Drosophila* consisting of the expression of the prion protein (PrP) from rodents, mainly hamster and mouse. Although rodents do not endure endemic prion diseases, they are excellent laboratory models of these conditions. In particular, hamsters played a critical role in the history of prion diseases because some strains develop fast, aggressive pathology (faster than in mice) and the larger brains produce large amounts of PrP needed for biochemical studies (Bolton et al. 1982). We started to work with rodent PrP for safety reasons: a "species barrier" prevents the transmission of mouse and hamster prions to humans. The species barrier is a well-known phenomenon that reveals the direct interactions mediating the templated conversion of natively folded "cellular" PrP (PrP^{C}) into pathogenic "scrapie" PrP (PrP^{Sc}) (Prusiner et al. 1998; Scheckel and Aguzzi 2018). Two independent labs created transgenic flies expressing hamster and mouse PrP either wild type or carrying mutations linked to familial forms of prion diseases in humans (Gavin et al. 2006; Fernandez-Funez et al. 2009). These models displayed multiple late onset disease-relevant features, including progressive locomotor dysfunction, degeneration of the architecture of brain circuits, and misfolding and aggregation of PrP. We have used these models in multiple studies comparing the intrinsic properties of WT PrP from animals sensitive and resistant to prion diseases, including hamster, mouse, rabbit, horse, and dog (Sanchez-Garcia et al. 2016; Fernandez-Funez et al. 2010; Sanchez-Garcia and Fernandez-Funez 2018). We have also introduced point mutations to these sequences to determine the consequences of altering key domains in an attempt to learn more about the sequence/structure relationships in PrP and the causation of disease. However useful, these *Drosophila* models of prion diseases had a key limitation: they show no eye phenotype in either young or aged flies. This is critical because the fly eye offers opportunities for many research applications, including comparisons of multiple constructs and unbiased genetic screens. What were our options for creating new models of prion diseases with eye phenotypes?

The first approach we discarded was introducing pathogenic mutations. These mutants had no significant differences from WT mouse and hamster PrP models. It became clear that trying other mutations would be futile in the context of rodent PrP backbone. The two strategies we considered were boosting PrP expression and shifting to PrP from animals with endemic prion diseases in which PrP shows a higher propensity to acquire neurotoxic conformations. Boosting expression alone by genetic tricks would not be enough because a hamster line carrying the P102L mutation was expressed 2.5× more than our strongest WT line and still had no eye phenotype (PFF, unpublished data).

We were left with one last option: introducing PrP from ruminants or human. Sheep, goats, and several cervids bear endemic prion diseases, whereas bovine developed "mad cow" disease after exposure to bone meal from contaminated sheep. We considered the introduction of PrP from these animals into flies, but we

also considered the advantages of introducing the human PrP. When comparing the diverse etiology and pathologies of endemic prion diseases, human prion diseases stand out for their variety. The most common form of the disease is sporadic Creutzfeldt-Jakob disease (CJD), which manifests initially as a dementia. Gerstmann-Sträussler-Scheinker (GSS) syndrome is an inherited prion disease that starts as a cerebellar ataxia, whereas fatal insomnia alters sleep patterns. These and other disorders illustrate the complex neuropathology of human prion diseases. It is striking to note the diversity of clinical presentations caused by a single protein, suggesting an intrinsic propensity to misfold into conformations with different neurotropisms (protein strains?). Thus, since we were considering introducing some risk in our work with PrP, we decided that the best option for generating new phenotypes was to generate flies expressing the human PrP.

Once we settled on introducing the human PrP, we considered the conditions that would boost its expression levels. Although it has not been used frequently, codon optimization is expected to boost gene expression by increasing translation efficiency. This increased translation can compensate for the fraction of the protein that is degraded early in biogenesis pathway due to misfolding in the ER. Thus, for our first attempt, we generated transgenic flies expressing codon-optimized human PrP from random insertions. This work was conducted in an insect room within a BSL3 facility (arthropod containment lab 3 or ACL3). When the transgenic flies were finally ready, we crossed them with the eye driver line *GMR-GAL4*. Voila!!! We had a new eye phenotype! The flies had disorganized, glassy eyes and the males had smaller eyes (Fernandez-Funez et al. 2017). They also showed another new phenotype that we had never observed with the rodent PrP lines: lethality with pan-neural expression (*Elav-Gal4*).

Following these initial encouraging results, we codon-optimized hamster, mouse, and bank vole PrP. Then, we inserted these constructs along with WT human PrP in the attP2 landing site. The bank vole is a recent model that can be used as the host for many different prions, suggesting the lack of a species barrier in these small rodents. We recently confirmed that the WT human PrP flies inserted in attP2 also display an eye phenotype, albeit slightly weaker than that in the original random insertions. Finally, we have used the new eye phenotype to complete an unbiased genetic screen for modifiers of PrP toxicity with excellent results. We have lots of work to do at this point to understand the gene networks implicated in PrP toxicity by using human.

PrP finally enabled us to conduct these studies. This is the first of its class in the field due to lack of models amenable to large genetic screens.

Therapeutic Rescue of Proteotoxicity

Hsp104 is a potent disaggregase in yeast and bacteria that plays an important role in stress survival and in the propagation of yeast prions (Shorter and Lindquist 2004). Hsp104 is part of a hexameric AAA+ (ATPase associated with diverse cellular

activities) protein with the ability to degrade insoluble amyloids by teasing apart monomers; it has been found to disaggregate diverse human amyloids in vitro, and in yeast, mice, and rats (Lo Bianco et al. 2008; Vacher et al. 2005; Satyal et al. 2000). Metazoans, including animals and humans, do not have an Hsp104 homologue but possess a different disaggregase, Hsp110, which cooperates with Hsp70 and Hsp40 to dissociate aggregates. Hsp104 is many orders of magnitude stronger compared to Hsp110, offering the potential of its novel therapeutic activity in animals. The recent generation of a fly model of neurodegeneration co-expressing Hsp104 offered the opportunity to exploit its protective activity under "therapeutic" conditions (Cushman-Nick et al. 2013).

Flies expressing SCA3-78Q were the first model of neurodegeneration published in *Drosophila* (Warrick et al. 1998). SCA3 is one of nine polyglutamine diseases in which a continuous polyQ tract causes misfolding and aggregation of a diverse group of host proteins. Expression of SCA3-78Q in the eye results in adult flies with disorganized, depigmented eyes and disorganized underlying retina. The retina continues to degenerate over several days resulting in loss of pigmentation and collapse of the eyes. The molecular chaperone Hsp70 was shown to robustly suppress the eye toxicity of SCA3-78Q despite not having an effect on its aggregation (Warrick et al. 1999). This protective activity is exerted when it is *expressed simultaneously* with SCA3-78Q owing to the ability of Hsp70 to refold misfolded monomers. The disaggregase activity of Hsp104 led authors to ask whether it would be capable of preventing further damage or even reversing degeneration if introduced *after the amyloid started to aggregate*. To answer this question, the authors had to develop new experimental approaches. The UAS/GAL4 system provides extraordinary experimental flexibility due to the availability of tens of thousands of transgenic flies expressing WT or RNAi alleles covering most *Drosophila* genes. But the predominant use of the UAS/GAL4 system comes with a price: multiple transgenes must be co-expressed simultaneously, with no opportunity to differentially control their temporal expression.

To split the control of SCA3-78Q and Hsp104, the authors introduced different expression systems for each construct. To express SCA3-78Q in the eye, they fused the minimal promoter of the *glass* gene (*glass multiple reporter*) to SCA3-78Q (*gmr1x-SCA3-78Q*) (Cushman-Nick et al. 2013). This fusion expresses SCA3-78Q constitutively in the eye at lower levels than *GMR-GAL4*/*UAS-SCA3-78Q* because the binary system results in an amplification step. This was not a big concern because SCA3-78Q has a robust eye phenotype and lower expression was still expected to result in abnormal eyes, and, in fact, a weak phenotype was preferable for its rescue under conditional Hsp104 expression.

The requirement for Hsp104 was to introduce robust temporal control independent from SCA3-78Q to examine its protective activity under therapeutic conditions (Cushman-Nick et al. 2013). First, the sequence of Hsp104 was codon-optimized to ensure robust translation. The UAS/GAL4 system exploits the temporal/spatial expression of particular regulatory sequences, but robust temporal control (switching on/off) is also possible through upgrades of the UAS/GAL4 system. TARGET takes advantage of a temperature-sensitive allele of the GAL4 inhibitor GAL80

(GAL80^{TS}). This system allows for tight temporal control but has practical disadvantages, like a shift in temperature that alters metabolic rate and aging, and the introduction of one additional genetic element. The GeneSwitch (GS) system is based on the expression of an engineered GAL4 activated by the steroid mifepristone. This makes the GS system simpler from a genetics point of view (only three transgenes needed). Among the concerns of the GS system are that adult flies eat little, resulting in weaker transgene expression, and non-specific expression (Cushman-Nick et al. 2013). Only a few GS driver lines exist at this time, but one of them is the *GMR-GS* that was ideally suited for this project. Given the robust protective activity of Hsp104, weak expression levels should still protect against SCA3-78Q toxicity.

So, how did this complex setup work out? Newly eclosed *gmr-SCA3-78Q* flies showed no external eye phenotype but had a disorganized retina (Cushman-Nick et al. 2013). This retinal phenotype progressed over the next 7 days to a loss of over 30% of the tissue. The eye phenotype of *gmr1x-SCA3-78Q* was weaker than expected, causing additional work to characterize the retina through sections. When Hsp104 was activated in 1-day-old adults, there was a significant amelioration of the retinal degeneration measured at day 7 compared to flies not expressing Hsp104. As controls, neither a catalytic-inactive Hsp104 nor WT Hsp70 rescued the retinal phenotype. It is also important to consider under these experimental conditions that there is a delay associated with feeding adult flies food containing mifepristone and the drug reaching its target in the eye. Considering these less than ideal conditions, this therapeutic activity of Hsp104 is very impressive.

When Hsp104 was activated on day 3, after SCA3-78Q had more time to accumulate and damage the retina, it was still able to ameliorate the degeneration of the retina, albeit less than when it was activated at day 1. These experiments are very impressive from a technical point of view and also reveal the extraordinary properties of Hsp104 to slow down the degenerative process that had already been initiated in the absence of the therapy. Interestingly, Hsp70 demonstrated no protective activity under the same conditions either at days 1 or 3 even though Hsp70 is a robust suppressor of SCA3-78Q when both are expressed simultaneously. Remarkably, the therapeutic protection by Hsp104 is not mediated by a reduction in SCA3-78Q aggregates, suggesting that binding of Hsp104 to large amyloid aggregates is protective for other reasons. This is similar to the previous description by N Bonini that Hsp70 rescued SCA3-78Q toxicity without altering its aggregation. We found similar results with an engineered Hsp70 that is secreted and suppresses Aβ toxicity via its chaperonin activity independently of its foldase activity (Fernandez-Funez et al. 2016). Overall, these results argue for a role of different molecular chaperones in preventing toxicity by mechanisms other than their disaggregase/foldase activity. While the relentless expression of amyloids in these chronic diseases overwhelms the proteostasis networks, the additional expression of Hsp104 or Hsp70 can recognize these aberrant structures of amyloids and prevent further damage. These results continue to build a strong argument for the therapeutic potential of several chaperones against protein misfolding disorders.

Astrocyte: Neuron Interactions Promoting Regeneration

The work discussed above introduced additional genetic elements to add temporal control in the expression of a therapeutic agent. The following example takes *Drosophila* genetics a step further by introducing two different *cell-specific* expression systems in the eye to understand the role of cell-to-cell interactions during degeneration (Li et al. 2018). Curiously, this paper uses the same disease paradigm: SCA3. In this case, the authors are interested in dissecting the contribution of glia and, in particular, astrocytes in the SCA3-78Q eye phenotype. For this paradigm, the authors introduce the relatively new Q-system (Riabinina et al. 2015; Potter et al. 2010). Dijkers and cols. were interested in understanding the contribution of astrocytes to SCA3-78Q-mediated neurodegeneration. For this, they devised a genetic screen in astrocytes for genes that modify SCA3-78Q toxicity in the eye. The main technical issue with this approach is the lack of transgenes cloned under the control of QUAS readily available. Thus, the logical choice was to dedicate the Q-system to the expression of SCA3-78Q and the flexibility of the UAS/GAL4 for selected candidate genes. The system already has an eye-specific driver line (GMR-QF2, second-generation QF) available, and the investigators created the SCA3-27Q and SCA3-78Q transgenes under the control of QUAS. For RNAi expression in astrocytes, the authors used the *alrm* (*astrocytic leucine-rich repeat module*)-*Gal4* line that is specifically expressed in astrocytes. The first interesting observation of the paper is that flies expressing SCA3-78Q, but not those expressing SCA3-27Q, recruit astrocytes to the retina as imaged by *UAS-RFP* (*alrm-Gal4*) (Li et al. 2018). Glia migrates into the developing retina from the optic lobe during normal eye development. The increase in RFP signal is likely a consequence of activation of astrocytes due to stress/cell death signals, resulting in an increase in size, elevated expression of RFP, and possibly recruitment of additional astrocytes to the retina.

Based on this reaction of astrocytes to the expression of SCA3-78Q, the authors investigated the involvement of candidate genes in this neuron/glia interaction. They focused on genes involved in neuron/astrocyte communication, including receptors, ligands, and intracellular factors from inflammatory pathways. RNAi lines for 156 genes revealed both enhancers and suppressors of the SCA3-78Q eye phenotype as measured by eye depigmentation and the number of necrotic spots (Li et al. 2018). Downregulation of NF-κB1 (encoded by *Drosophila Relish*) and other factors in the NF-κB1 signaling pathway suppressed SCA3-78Q toxicity. Interestingly, co-expression of SCA-78Q and *Relish-RNAi* in the eye did not result in attenuation of the SCA phenotype, thus indicating the non-autonomous role of *Relish* in astrocytes in the disease. The role of neuroinflammation in neurodegenerative diseases has been widely described in pathological samples, and many groups are currently pursuing studies to determine the functional role of neuroinflammation in disease progression.

The authors then use the knowledge gained in the eye to perform behavioral and survival experiments with two expression systems. In these experiments, SCA-78Q

was expressed pan-neuronally (not in astrocytes) by fusion of the activator, QF2, to the promotor of neuron-specific gene *nSyb*. *Relish-RNAi* expression was limited to astrocytes via the *alrm-GAL4* driver. To introduce temporal control and mimic a late-onset disease model, QS was then inhibited in adult flies by supplementing their food with quinic acid. Downregulation of *Relish* in these experiments led to increased motor function and survival (Li et al. 2018). The complexity of the experiment is such that it can only be approached after sufficient information has been gained in a more practical assay like the eye.

This work uniquely exemplifies the opportunities that *Drosophila* offers to innovative research. This work opens the door to a larger, unbiased screen for other glial genes mediating neurodegeneration in SCA3 and other amyloids.

Coupling Eye Structure and Function

We have argued that the main advantage of the *Drosophila* eye was the fast observations for dynamic experiments. But sometimes slow, careful observations can result in a treasure-trove of data. The fly eye is a highly organized structure composed of several hundreds of ommatidia, the visual unit of insects. Each ommatidia contains eight photoreceptors precisely arranged in an invariable pattern; slight changes in this structure can be visible in intact eyes or revealed through histological sections. Histological sections are powerful, particularly ultrathin sections analyzed under the transmission electron microscope (TEM), but they are time-consuming. Furthermore, photoreceptors are highly specialized neurons that can produce action potentials when stimulated by light. The electrical activity of photoreceptors can be measured by electroretinography (ERG) to reveal functional aspects of the eye not linked to morphological changes. How about combining ERG and TEM to analyze the eye phenotype of three *Drosophila* models of neurodegenerative diseases at three different time points? No way!

In 2016, a paper did exactly that!! The authors describe the characterization of *Drosophila* models of Parkinson's and Alzheimer's disease expressing α-synuclein (α-syn), tau, or Aβ42 (Chouhan et al. 2016). To focus exclusively on the effect of these genes on photoreceptors, they expressed the transgenes under the control of *rhodopsin-1* promotor (*Rh1-Gal4*) instead of using the more common *GMR-Gal4*, which is expressed in all eye cells. *Rh1* is expressed in mature photoreceptors, preventing some of the developmental perturbations induced by the toxicity of the three amyloids. To further restrict their toxicity during development, the authors grew the crosses at 18 °C and shifted the flies to 25 °C after eclosion of adult flies. Under these conditions, the retinas of young flies expressing α-syn, tau, or Aβ42 display normal size, organization, and electric activity. Continuous monitoring of ERG in flies during aging indicates that flies expressing α-syn and tau undergo dramatic changes in the generation of action potentials, with reduced amplitudes and other changes (Chouhan et al. 2016). Surprisingly, expression of Aβ42 showed no

significant changes in ERG over 20 days. When looking at the eye retina, both Aβ42 and α-syn demonstrated dramatic degeneration over 20 days, whereas tau did not.

Finally, examination of the structure of photoreceptors at higher resolution showed that α-syn and tau underwent progressive degeneration of rhabdomeres, the membranous structure where photosensitive pigments accumulate, whereas Aβ42 did not (Chouhan et al. 2016). All three models showed an increase in the accumulation of autophagic vesicles, with Aβ42 and α-syn showing higher levels than tau. In contrast, tau flies accumulated more electron dense vesicles. These results show that tau, Aβ42, and α-syn show different neurodegenerative phenotypes in photoreceptors. This paper highlights the power of *Drosophila* for conducting highly detailed studies, including functional and structural analysis of photoreceptors. Despite the time-consuming techniques employed in this paper, the information extracted is very novel and highly relevant toward understanding the mechanisms underlying neurotoxicity of amyloids and the differences between amyloids.

Concluding Remarks

In this chapter, we reviewed a few recent examples of the impact of new technologies and experimental approaches to study neurodegeneration in the *Drosophila* eye. Some challenges deal with the optimization of the models to push for eye phenotypes (human PrP), whereas others deal with the problem of comparing multiple constructs (DPRs, Aβ). These examples draw on the experimental flexibility of *Drosophila* to develop appropriate solutions, including codon optimization and common attP landing sites. These two examples make use of several available genetic tools to answer complex questions that involve expressing two sets of constructs at different times or in different cell types. The eye plays a key role as an easily accessible structure that facilitates the observation and scoring of genetic interactions that provide new insight into disease pathogenesis or therapy. On the other hand, the complexity of the eye as a peripheral organ of the nervous system provides access to morphological and physiological studies that reveal profound details about the consequences of expressing toxic biological agents involved in human disease. Finally, overall, these examples demonstrate the power of *Drosophila* as a research tool due to the rich technical resources made available by a collaborative and sharing community.

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Genetic Regulation of Early Eye Development in Non-dipteran Insects

Markus Friedrich, Ying Dong, Zhenyi Liu, and Iris Yang

Comparative analyses of eye development in *Drosophila* and distantly related phyla have fundamentally changed the way we think about the evolution of animal eyes today. On the one hand, it is clear that select eye patterning mechanisms have deep evolutionary roots, such as the involvement of *Pax6* and an ever-extending catalogue of additional transcription factors with selector gene-like functions in development (Donner and Maas 2004; Gehring 2002; Kozmik 2008; Pichaud and Desplan 2002). On the other hand, the diversity of distinct eye types in extant animals implies the evolution of lineage-specific patterning processes, superimposed onto ancient gene interactions inherited from the prototype eye at the dawn of animal evolution (Lamb 2011; Nilsson 1996; Salvini-Plawen and Mayr 1977; Zuker 1994). It is therefore an important question to explore how far back the regulatory program organizing the development of the compound eye in *Drosophila* can be traced in arthropod evolution.

Elaborate compound eyes are found in living representatives of all arthropod phyla, which include crustacea, chelicerates, and myriapods in addition to insects (Buschbeck and Friedrich 2008; Fahrenbach 1969; Müller et al. 2003). The oldest fossils of advanced compound eye design have been discovered in deposits of the

M. Friedrich (✉)
Department of Biological Sciences, Wayne State University, Detroit, MI, USA

Department of Anatomy and Cell Biology, Wayne State University, Detroit, MI, USA
e-mail: mf@biology.biosci.wayne.edu

Y. Dong
Harold C. Simmons Cancer Center, UT Southwestern Medical Center at Dallas, Dallas, TX, USA

Z. Liu
Washington University, St. Louis, MO, USA

I. Yang
Department of Biological Sciences, Wayne State University, Detroit, MI, USA

A. Singh, M. Kango-Singh (eds.), *Molecular Genetics of Axial Patterning, Growth and Disease in Drosophila Eye*,
https://doi.org/10.1007/978-3-030-42246-2_11

early Cambrian, dating 515 million years before present (Lee et al. 2011; Paterson et al. 2011). The regulatory programs patterning the *Drosophila* compound eye retina may thus be hundreds of millions of years of age. Comparative studies in arthropods therefore offer unique opportunities to dissect the conserved and evolutionary younger components in the genetic control networks which pattern the *Drosophila* eye. To this end, a number of gene-specific studies have been carried out in representatives of other arthropod phyla, such as crustaceans and the horseshoe crab *Limulus polyphemus*, the only extant chelicerate with compound eyes (Blackburn et al. 2008; Duman-Scheel et al. 2002; Smith et al. 1993). Also the cellular organization of growth and differentiation of the visual system has been studied in non-insect arthropods (Hafner and Tokarski 1998, 2001; Harzsch and Walossek 2001; Melzer et al. 2000). However, the arguably most comprehensive comparative molecular studies of compound eye development have thus far focused on non-dipteran insect species up to this point.

Here, we introduce the satellite model organisms which have been used in comparative genetic studies of insect compound eye development and their phylogenetic relationships. This is followed by a review of the molecular findings that concern the specification of the retinal precursor tissues in these organisms. The cellular assembly of retinal precursor cells in the differentiating retina is strongly conserved in arthropods and has been previously reviewed in depth (Buschbeck and Friedrich 2008; Friedrich et al. 2006) and will thus not be further explored here.

The Phylogenetic Framework

The number of non-dipteran species that have been studied with comparative questions regarding the developing compound eye is still dwindlingly small against the backdrop of insect diversity (Fig. 1). Besides studies looking at the morphogenesis of very unusual visual systems, such as stalk-eyed flies or the enigmatic Strepsiptera (Buschbeck 2005; Buschbeck et al. 2001), molecular work boils down to five species. Two of these belong to the same ancient order of hemimetabolous insects: the Orthoptera. This includes the two-spotted cricket *Gryllus bimaculatus* and the American desert locust *Schistocerca americana*. However, *G. bimaculatus* belongs to the suborder Ensifera, while *S. americana* is part of the second orthopteran suborder Caelifera.

Orthoptera is 1 of the 22 currently recognized direct-developing insect orders, which form most adult body structures during embryogenesis. Adult size and finalized body plan are reached during the postembryonic growth stages of juvenile, i.e., nymphal instars. Except for wing and genital appendages, the body plan organization of nymphal instars is usually equipped with all essential structures of the adult form (Truman and Riddiford 2002). The Orthoptera are considered to have split at least 350 million years ago from the lineage that eventually gave rise to the ancestor

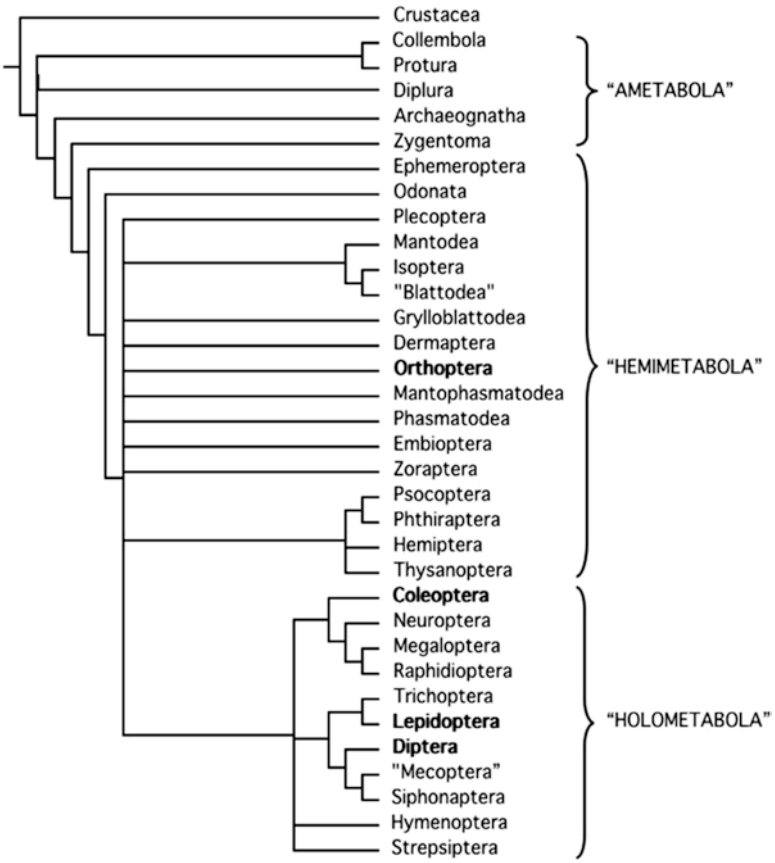

Fig. 1 Phylogenetic framework. Bold groups include model system used in studies of insect eye development. Quotation marks indicate paraphyletic groups. Ametabolous insects are primitively wingless and undergo less postembryonic changes than hemi- and holometabolous forms. Adapted from Friedrich et al. (2006)

of the large superclade of endopterygote or holometabolous insects, which transition through larval growth stages and the pupal resting stage before acquiring adult morphology (Beutel et al. 2011; Kristensen 1999) (Figs. 1 and 2).

Besides *Drosophila*, holometabolous insects include three further significant models of insect eye development: the red flour beetle *Tribolium castaneum*, the silk moth *Bombyx mori*, and the tobacco hornworm *Manduca sexta*. As a representative of the Coleoptera (beetles), *Tribolium* represents one of the oldest orders in the Holometabola, while silk moth and tobacco hornworm, as representatives of the order Lepidoptera, are more closely related to the order of true flies (Diptera) and thus *Drosophila* (Beutel et al. 2011; Kristensen 1999; Wiegmann et al. 2009).

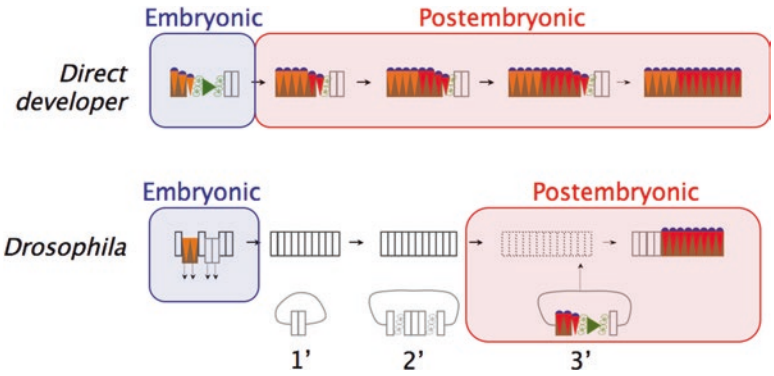

Fig. 2 Homology of embryonic and postembryonic visual system development between direct-developing insects and *Drosophila*. Conceptual alignment of homologous phases of visual system development in the direct-developing species and the holometabolous *Drosophila*. In direct-developing species, ommatidia develop during both embryogenesis (blue backdrop shade) and postembryogenesis (red backdrop shade). Ommatidia of both embryonic (orange cell bodies) and postembryonic (red cell bodies) origin become part of the adult eye. In *Drosophila*, the development of the visual system is split in two discrete phases. The embryonic phase produces larval eyes, which are not integrated into the adult eye. The postembryonic phase begins with the initiation of retinal determination and differentiation in the eye-antennal imaginal disc of the third (3′) larval instar. As a result, the adult *Drosophila* eye consists entirely of postembryonic ommatidia. The eye-antennal disc precursor disc tissue separates from the larval epidermis during embryogenesis and experiences continued growth during the first (1′) and second (2′) larval instar. During metamorphosis, the eye-antennal imaginal disc derivatives completely replace the larval epidermis during pupation. Apoptosis of larval epidermis indicated by dotted outlines. Color code of cellular components: gray = epithelial cells which persist from the embryo into adult, black = epithelial cells which are disposed during postembryogenesis, dark blue = cone cells, brown = pigment cells, orange cones = embryonic photoreceptor cells, red cones = postembryonic photoreceptor cells, filled orange circles = internalized larval eyes, green = mitotic cells. Progressing front of retinal differentiation represented by forward pointing green arrowhead

Adult Compound Eye Development in Direct- Versus Indirect-Developing Insects

Prerequisite to the comparison of compound eye development between direct-developing species and the indirect-developing *Drosophila* he clarification of homology relationships between specific phases of eye development, which are not obvious at first glance (Fig. 2). In direct-developing species, a significant part of the adult compound eye differentiates already in the embryo. As a result, about 20% of the posterior adult compound eye is usually of embryonic origin in direct-developing species. The anterior portion, by contrast, is added on during postembryonic development (Friedrich 2006). This mode of compound eye development is typical of direct-developing insects where larval and adult form shows relatively mild body plan differences.

Importantly, although the embryonic phase of eye development contributes to structures of the adult eye in direct-developing species, this developmental process

is not homologous to the entirely postembryonic development of the adult eye in the *Drosophila* eye disc. The latter corresponds, instead, exclusively to the postembryonic phase of compound eye development in direct-developing insects (Fig. 2). The embryonic phase of compound eye development in direct-developing species is homologous to the embryonic development of the larval eyes of holometabolous insects such as the *Drosophila* Bolwig organs (Friedrich 2013). These relationships are supported by comparative developmental and molecular evidence and have important consequences for the comparison of retinal primordium patterning mechanisms (Friedrich 2006, 2008).

Direct-developing insects differ from holometabolous insects like *Drosophila* also with respect to the transition from embryonic to postembryonic visual development. In direct-developing insects, this transition proceeds with the continued expansion of the nymphal compound eye that has been formed in the embryo. In holometabolous insects, however, the development of the larval and adult eyes is temporally and spatially separate process (Fig. 2).

It has been hypothesized that the developmental evolution of this separation began with the transient arrest of retinal differentiation during postembryonic development (Dong and Friedrich 2010). In support of this, a transient arrest of retinal differentiation can be enforced by the manipulation of eye developmental regulators in direct-developing insects like grasshopper (Dong and Friedrich 2010). Of note, the transient arrest model of biphasic eye development evolution is also consistent with the intermittent developmental arrest of other organs in the larval stage of holometabolous insects such as the leg appendages (Singh et al. 2007; Suzuki et al. 2009).

The American Desert Locust *Schistocerca americana*

The American desert locust and the closely related African desert locust *Schistocerca gregaria* have a long history of serving as experimental models in developmental and neurobiological research due to the accessibility of neural elements in both the embryo and the adult form (Moreaux and Laurent 2007; Rogers et al. 2010; Sanchez et al. 1995). Building on this body of work, the grasshopper system has also been adopted for the comparative developmental analysis of insect segmentation (Dearden and Akam 2000), appendage development (Mahfooz et al. 2004), and the development of the visual system (Dong and Friedrich 2005, 2010).

Organization of the Grasshopper Retina

Desert locusts are famous for their voracious food consumption, large body size, and coordinated long-distance flights (Lomer et al. 2001). These key traits of a major pest species are supported by an enormous visual system. First instar grasshopper nymphs hatch with compound eyes of close to 2500 ommatidia (Anderson

1978). This number increases to approximately 9,400 in the adult eye by the addition of new ommatidia at the anterior margin of the eye during postembryonic development which proceeds through five to six nymphal instars (Dong and Friedrich 2010). Grasshopper ommatidia contain a canonical set of 8 photoreceptor cells, 4 cone cells, and 2 primary pigment cells, which are surrounded by 16 secondary pigment cells (Wilson et al. 1978). The photoreceptor cells exhibit three morphological subtypes. There are two photoreceptors with proximally restricted rhabdomeres, five photoreceptors with rhabdomeres extending along the entire proximodistal axis of the ommatidium, and a single photoreceptor with a distally restricted rhabdomere that corresponds to the *Drosophila* R7 cell (Wilson et al. 1978). Electrophysiological data suggest the presence of green-, blue- and UV-sensitive photoreceptors (Bennet et al. 1967; Vishnevskaya et al. 1985). Homologs of the green- and UV-sensitive opsin gene families have been identified (Towner et al. 1997), but their spatial expression patterns, which would shed further light into the functional organization of photoreceptor subtypes in the grasshopper eye, are still unknown.

It is therefore also not yet known whether the grasshopper retina is subdivided into specialized subcompartments. There is, however, a detailed analysis of the retinal organization of the distinct dorsal rim area (DRA) at the dorsal margin of the eye that is populated with anatomically specialized photoreceptor cells (Homberg and Paech 2002). The DRA is a polarized light-sensitive compartment of the insect eye, whose development and function have been studied in detail in *Drosophila* (Labhart and Meyer 1999; Wernet et al. 2012).

Embryonic Phase of Grasshopper Eye Development

The embryogenic development of grasshopper species like *Schistocerca* takes about 20 days, i.e., advances approximately 5% per day (Bentley et al. 1979). At about 20% of embryogenesis, the grasshopper embryo has formed a distinct head region with two prominent lateral extensions: the head lobes. The posterior region of the head lobes will transform into a secondary set of lobe-like compartments that are exclusively occupied by precursor tissue of the visual system, likely homologous to the visual anlagen in the *Drosophila* embryo (Chang et al. 2001). These compartments are the eye lobes (Fig. 3a) (Dong et al. 2003; Roonwal 1936). The outermost epithelial layer of the eye lobes constitutes the precursor tissue, i.e., primordium, of the retina. In addition, the eye lobes house the developing outer and inner optic neuropiles: lamina, medulla, and lobula (Dong et al. 2003).

Retinal differentiation initiates between 30% and 35% of embryonic development like *Schistocerca*, leading to the formation of a morphogenetic furrow-like front of differentiation, which travels the eye lobe ectoderm from posterior to anterior (Fig. 3b and f).

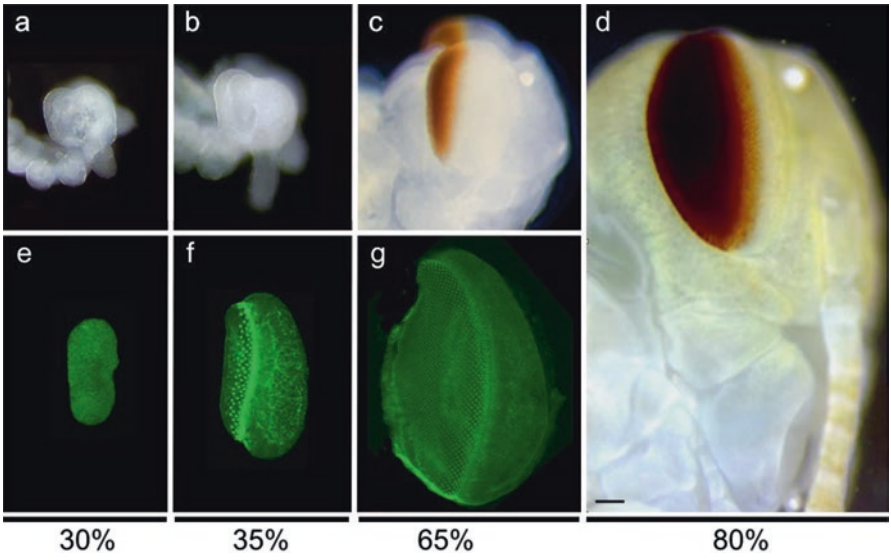

Fig. 3 Embryonic eye development in the grasshopper *S. americana*. (**a**–**d**) Lateral stereomicroscopy view of embryonic head at (**a**) 30%, (**b**) 35%, (**c**) 65%, and (**d**) 80% of embryonic development. (**e**–**g**) Laser scanning confocal images of differentiating embryonic retina labeled with phalloidin, which highlights cell morphogenesis by binding to f-actin, at respective stages of development. A morphogenetic furrow-like differentiation front can be seen starting from 35% of development (**f**)

Co-expression of sine oculis and eyes absent in the Grasshopper Embryonic Eye Lobes

The transcription factor genes *eyes absent* (*eya*) and *sine oculis* (*so*) represent the earliest markers of the visual anlage in the *Drosophila* embryo, a neuroectodermal field in the median head that contains the precursor cells of the entire visual system (Chang et al. 2001). Consistent with a conserved function of *eya* and *so* in the specification of the embryonic visual anlagen, the *Schistocerca* orthologs of *so* and *eya* are co-expressed in the periphery of the head lobes and thus soon after gastrulation (Dong and Friedrich 2005) (Fig. 4a and d). As the eye lobes emerge, *eya* and *so* continue to be strongly co-expressed in the retina, lamina, and medulla tissue layers (Fig. 4b and c, e and f).

After the initiation of retinal differentiation, *eya* and *so* are detected throughout the differentiating retina and the morphogenetic furrow as well as in a wide area of the undifferentiated neuroectoderm ahead of the morphogenetic furrow (Fig. d and f). This *eya* and *so* co-expressing field ahead of the furrow appears limited to a range defined by its distance from the morphogenetic furrow. This observation and the gradient-like decrease of the *eya* and *so* expression levels toward the anterior margin of their co-expression domain have been taken as circumstantial evidence that the expression of *eya* and *so* may be primarily transcriptionally activated by signals

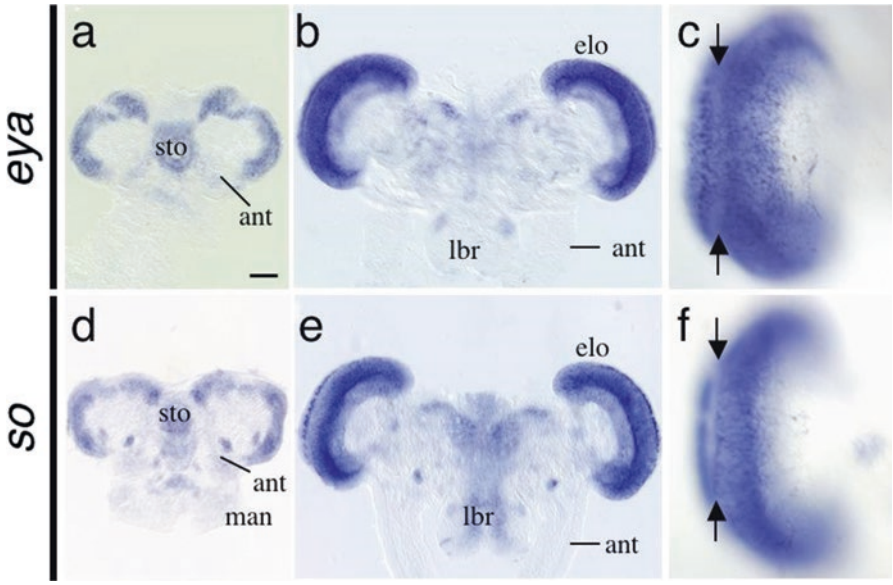

Fig. 4 Expression of *eya* and *so* in the grasshopper eye lobes. (**a**, **b**, **d**, **e**) Frontal view of grasshopper embryonic head. Dorsal up. (**c**, **f**) Optical section of eye lobe from lateral perspective at the level of the peripheral ectoderm. (**a**–**c**) Specimens labeled by whole mount in situ hybridization for transcript detection of *eya* (**a**–**c**) and *so* (**d**–**f**). Black arrows indicate retinal front of differentiation. Dorsal up and anterior to the right. Abbreviations: ant = antenna, elo = eye lobe, lbr = labrum, man = mandible, sto = stomodeum

emanating from the morphogenetic furrow in a manner comparable to the induction of the preproneural (PPN) field in the *Drosophila* eye disc (Bessa et al. 2002; Dong and Friedrich 2005; Greenwood and Struhl 1999).

In *Drosophila*, the PPN field is activated through long-distance signaling by the transforming growth factor-β homolog *decapentaplegic* (*dpp*). This function of *dpp* is associated with the strong and specific expression in the morphogenetic furrow (Heberlein et al. 1993). In the grasshopper, however, *dpp* is not expressed in the morphogenetic furrow (Friedrich and Benzer 2000). Instead, a low transcript level of *dpp* is detected throughout the anterior eye lobe ectoderm ahead of the morphogenetic furrow (Fig. 8). While *dpp* may function in this domain as a growth activating factor, at face value this pattern is not consistent with a furrow movement organizing function like in the *Drosophila* eye-antennal disc. Extending the *Drosophila* paradigm, the signaling factor *hedgehog* (*hh*) could be an alternate candidate inducer of the PPN expression domain in the grasshopper (Heberlein et al. 1993; Ma et al. 1993). While this possibility remains to be explored in the grasshopper, this scenario is supported by the reported expression of *hh* in the embryonic eye lobes of crickets (see below) (Niwa et al. 2000).

Expression and Function of wg

Investigations of the expression of *wingless* (*wg*) in the grasshopper have produced evidence that this signaling factor functions as an antagonist of *eya* and *so* very similar to the situation in the anterior eye-antennal disc of *Drosophila* (Dong and Friedrich 2005; Pichaud and Casares 2000). In the embryonic eye lobe, *wg* is expressed in two prominent polar domains (Friedrich and Benzer 2000; Liu et al. 2006), which appear to be clear of *eya* and *so* (Fig. 5). In *Drosophila*, *wg* acts as growth activator in the anterior *Drosophila* eye disc, repressing the onset of precocious retinal differentiation (Baonza and Freeman 2002; Lee and Treisman 2001; Treisman and Rubin 1995). A hypothesized repressive effect of *wg* on retinal specification in the grasshopper was tested by LiCl incubation experiments with cultured embryonic eye discs (Dong and Friedrich 2005). Through its inhibition of glycogen synthase kinase 3β, LiCl application is known to stimulate Wg signaling (Stambolic et al. 1996). In the cultured eye lobes, the addition of LiCl caused a stalling of retinal differentiation. This was associated with a strong increase of cell division anterior to the morphogenetic furrow and cell death posterior to the morphogenetic

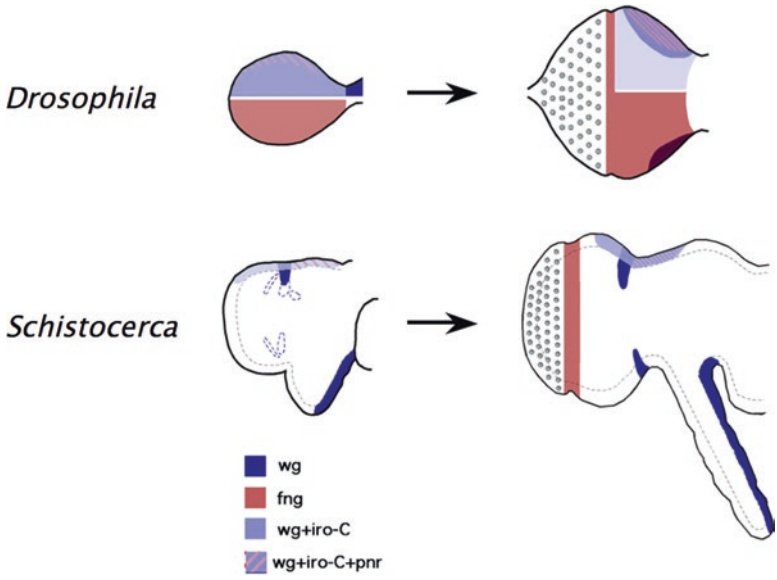

Fig. 5 Dorsoventral patterning gene expression in *Drosophila* and grasshopper. Schematic comparison of the expression domains of *wg* and *fng* as well as areas with overlapping expression of *wg* with *Iro-C* or *wg* and *Iro-C* and *pnr*. Left column shows the *Drosophila* eye disc and the grasshopper head hemisphere at an early developmental stage that precedes the onset of retinal differentiation (second larval instar eye-antennal imaginal disc in *Drosophila* and 30% stage of *Schistocerca*). The right column compares the late third larval instar eye imaginal disc of *Drosophila* with the left grasshopper head hemisphere at about 45% stage of *Schistocerca* embryo dorsal up and anterior to the right. Adapted from Dong and Friedrich (2005)

furrow (Dong and Friedrich 2005). These findings are consistent with the role of *wg* as growth activator in the anterior *Drosophila* eye disc and its impact on differentiation in the posterior *Drosophila* eye disc (Baonza and Freeman 2002; Lee and Treisman 2001; Treisman and Rubin 1995), suggesting deeply conserved functions of *wg* in the control of retinal patterning in insects.

Dorsoventral Patterning

In *Drosophila*, the activation of focal notch (N) signaling along the midline of the early eye disc is essential for stimulating the rapid expansion of the eye primordium by cell proliferation (Cho and Choi 1998; Dominguez and de Celis 1998; Dominguez et al. 2004; Kenyon et al. 2003; Papayannopoulos et al. 1998). In addition, the differential expression of N-signaling components in the dorsal and ventral halves of the eye disc anticipates the compartmentalization of the adult eye into dorsoventral compartments (Reifegerste and Moses 1999). Together with *wg*, the analysis of the expression of the grasshopper homologs of the N-signaling modifier glycosyltransferase *fringe* (*fng*) and the transcription factor genes *Delta* (*Dl*), *pannier* (*pnr*), and *Iroquois-C* (*Iro-C*) provided insights into the dorsoventral patterning organization of the grasshopper eye (Dong and Friedrich 2005).

Similar to the *Drosophila* situation (Cavodeassi et al. 1999, 2000; Maurel-Zaffran and Treisman 2000), *pnr* and *Iro-C* are expressed in dorsal populations of the embryonic head. However, in contrast to *Drosophila*, the expression of *pnr* remains outside the eye lobes, representing an extension of the dorsal margin cells. Further, the expression of *Iro-C* extended only 10% into the dorsal pole of the anterior embryonic eye lobe, consistent with a role in patterning the grasshopper DRA ommatidia but incompatible with a role in subdividing the retina field into a dorsal and ventral half. In combination, these data seem to suggest that conserved genetic mechanisms in DRA specification exist, but not, however, with respect to the dorsoventral patterning in the retina of grasshopper and *Drosophila* (Fig. 5). In further support of this latter notion, the expression of *Dl* and *fng* shows also no evidence of dorsoventral compartmentalization ahead of the morphogenetic furrow or prior to its initiation in the grasshopper embryonic eye lobe (Fig. 5) (Dong and Friedrich 2005).

The Postembryonic Phase of Grasshopper Eye Development

In direct-developing insects like *Schistocerca*, the retinal precursor cell population of the anterior eye lobe neuroectoderm transforms into a growth zone margin during the transition from embryonic to postembryonic development, ultimately outlining the anterior edge of the nymphal eye (Figs. 2 and 6a and b) (Dong et al. 2003; Friedrich 2006). The cellular organization of this retinal growth zone, which is

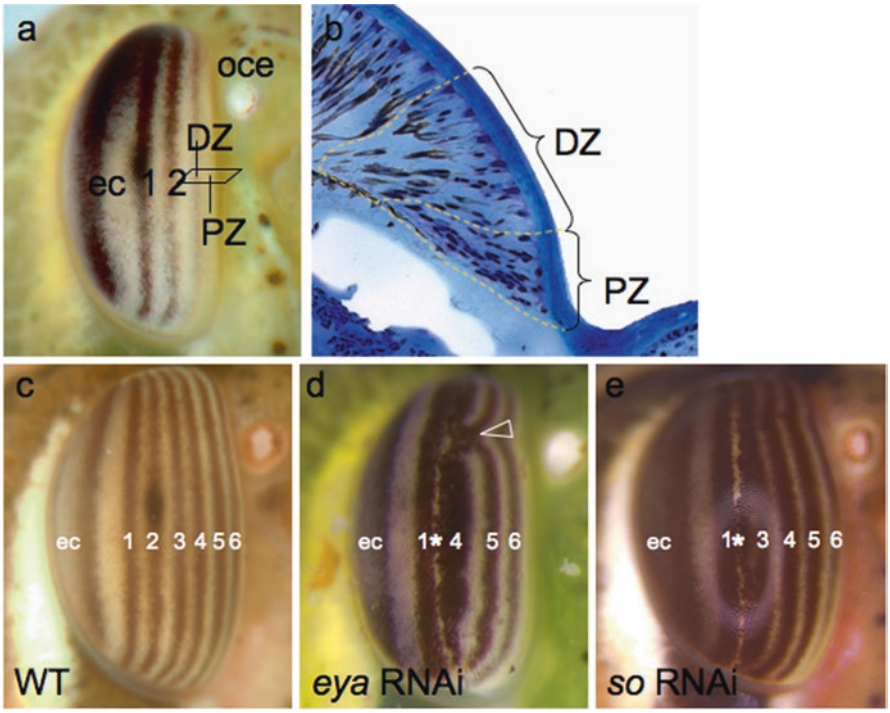

Fig. 6 Effect of eya and so knockdown on the postembryonic development of the grasshopper compound eye. (**a**) Frontolateral view of fourth instar grasshopper nymphal eye. Relative position of differentiation zone (DZ) and proliferation zone (PZ) is indicated and related to section plane of panel b. The posterior dark pigmented region of the eye that is generated in the embryo is labeled as embryonic cap (ec). Numbers label pigment stripe areas formed during postembryonic retina differentiation in the first two nymphal instars. (**b**) Toluidine blue-stained sagittal semithin section through the anterior compound eye of a first instar grasshopper nymph. Cells in the DZ elongate and accumulate pigment. Cells in the PZ are densely packed and undifferentiated. (**c**–**e**) Lateral view of adult compound eye. (**c**) Untreated wild-type animal. (**d**) Strongly affected *eya* knockdown animal. Asterisk in panel **e** indicates position of scar between stripes 1 and 4. Arrowhead in e points at disrupted anterior stripe pattern. (**e**) Phenotypic *so* knockdown animal. Asterisk indicates position of scar between stripes 1 and 3. In all panels anterior is to the left and dorsal up. ec = embryonic cap. Numbers identify specific lateral pigment stripes. Abbreviations: gen = gena, oce = ocellus. Adapted from Dong and Friedrich (2010)

heavily enriched with mitotic cells, has been described in early histological studies (Anderson 1978; Bodenstein 1953). Today, it is interesting to note its similarity to the ciliary margin region of the fish or amphibian eye (Perron et al. 1998; Raymond et al. 2006). Posterior to the proliferation zone, the transition into the fully differentiated retina is filled with intermediate stages of ommatidial development defining the differentiation zone (Fig. 6b) (Anderson 1978; Dong and Friedrich 2010).

Unfortunately, the molecular organization of the grasshopper eye proliferation zone is still little investigated. Gene knockdown experiments, however, targeting *eya* and *so* mediated by RNA interference (RNAi) produced first insights into the function

of eye selector genes during postembryonic eye development in the grasshopper (Dong and Friedrich 2010). Knocking down *eya* or *so* leads to a transient arrest of postembryonic retina differentiation in nymphs which completed development into the adult form, generating adult eyes with a pronounced vertical scar area (Fig. 6). These findings were interpreted to suggest that the downregulation of *so* and *eya* does not irreversibly affect the organization of the mitotic activity in the growth zone (Dong and Friedrich 2010). Thus, *eya* and *so* have been proposed to act in a similar manner in the postembryonic grasshopper eye, as in the PPN zone of the *Drosophila* eye disc, making cells responsive and competent to undergo retinal differentiation.

The Two-Spotted Cricket *Gryllus bimaculatus*

Driven by major efforts to develop tools for molecular analysis, such as whole mount in situ hybridization, RNAi-mediated gene knockdown, and germline transformation, the cricket *G. bimaculatus* has evolved into a versatile and efficient model system for comparative development (Fig. 7) (Horch et al. 2017). Crickets are generally crepuscular and less prominent in the areal insect fauna. Consistent with this, crickets do not exhibit flight behavior under laboratory conditions unless artificially stimulated. And yet, female crickets perform extensive prereproductive flight dispersal (Lorenz 2007), a capacity which is associated with a pronounced visual system.

Organization of the Cricket Retina

The adult eye of *G. bimaculatus* consists of approximately 4,600 ommatidia (Labhart and Keller 1992). Like in the grasshopper, this includes a structurally and functionally distinct DRA, which is populated by blue- and UV-opsin-expressing

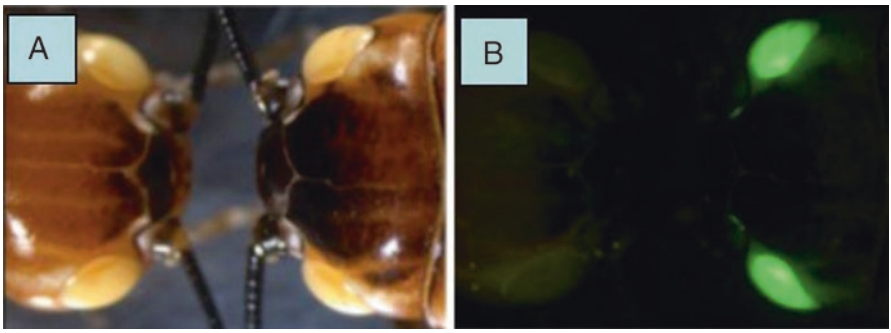

Fig. 7 Eye morphology of the cricket *Gryllus bimaculatus*. (**a**) Stereomicroscope view of dorsal head of white-eyed wild-type (left) and transgenic (right) animal. (**b**) Epifluorescence image of the same, note strong EGFP expression in the compound eye of the transgenic animal. Kindly provided by Dr. Sumihare Noji

photoreceptors (Blum and Labhart 2000; Henze et al. 2012). The analysis of opsin gene expression patterns uncovered further compartmentalization (Henze et al. 2012). The main retina encompasses a blue- and green-opsin-expressing ventral area, while the dorsal remainder expresses UV- and green-opsin.

Patterning Gene Expression and Function During the Embryonic Phase of Cricket Eye Development

The early developing cricket visual system is organized in the same way as the eye lobe compartments in grasshoppers (Inoue et al. 2004). Likewise in correspondence to the organization in the grasshopper, retinal differentiation is initiated in the posterior margin of the eye lobe ectoderm, and a morphogenetic furrow-like front of differentiation travels the cricket eye lobe neuroectoderm in posterior to anterior direction (Inoue et al. 2004; Takagi et al. 2012).

The available expression data on the cricket homologs of *wg*, *hh*, and *dpp* suggest that *wg* is expressed in the anterior margins of the eye lobe, while *hh* and *dpp* are expressed in different dorsoventral domains across the eye (Fig. 8) (Niwa et al. 2000). *hh* in particular appears to be strongly expressed in the differentiating retina (Niwa et al. 2000). At face value, these data are consistent with conserved roles of *dpp* and *hh* in promoting eye development and a conserved function of *wg* as tissue growth-stimulating antagonist of retinal differentiation (Friedrich 2006; Liu et al. 2006).

With respect to transcription factors, the expression patterns of *so* and *eya* as well as *dachshund* (*dac*) have been studied in detail (Fig. 8) (Inoue et al. 2004; Takagi et al. 2012). The expression of *dac* is detected in the eye lobe neuroectoderm prior to morphogenetic furrow initiation (Inoue et al. 2004). In the differentiating eye, *dac* transcript levels are concentrated in the morphogenetic furrow and below detection level both anterior and posterior to the morphogenetic furrow (Inoue et al. 2004).

The orthologs of *so* and *eya* in the cricket are strongly expressed in the non-differentiated area of the eye lobes prior to the initiation of eye differentiation

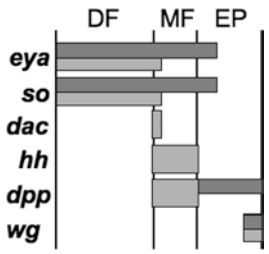

Fig. 8 Summary of eye developmental expression patterns in orthopteran species. Gray, expression domain in cricket; black, expression domain in grasshopper. Abbreviations: DF = differentiating retina, EP = eye primordium, MF = morphogenetic furrow

(Takagi et al. 2012). Thereafter, *so* and *eya* expression extends from the morphogenetic furrow uniformly across the differentiating retina in the posterior head lobe, much the same as in grasshopper. However, the expression of *so* and *eya* seems more confined anterior to the morphogenetic furrow, raising the possibility of differences in the transcriptional organization of retinal induction between the two species (Fig. 8). Consistent with a predicted function of *eya* in specification and differentiation of the eye during embryonic development, the parental RNAi-mediated knockdown of *eya* resulted in strong eye depletion phenotypes, including complete loss (Takagi et al. 2012).

Expression and Function of eya and so During the Postembryonic Phase of Cricket Eye Development

The role of *eya* and *so* has also been studied in the nymphal eye of *G. bimaculatus* (Takagi et al. 2012). This analysis revealed the presence of defined anterior proliferation and differentiation zones similar to the nymphal eye of grasshopper. Moreover, expression analysis of *eya* revealed the differential accumulation of transcripts in the proliferation zone and posterior to it, in both differentiating and differentiated pigment cells (Takagi et al. 2012). The RNAi-mediated knockdown of *eya* or *so* by dsRNA injection into third instar nymphs resulted in highly informative phenotypes. In the strongest *eya* knockdown animals, the proliferation zone appeared completely missing. Moreover, the posterior retina region of the cricket, which had differentiated prior to injection, reorganized into a non-sensory head cuticle (Takagi et al. 2012).

While these data are consistent with the expected role of *eya* in specification and differentiation of the postembryonic cricket eye, the mechanism explaining its role in the maintenance of differentiated retinal cell states will require further investigation. In contrast to grasshopper, the data suggest that *eya* and *so* are essential not only for the differentiation of the nymphal retina but also for the maintenance of the proliferation zone.

Comparing *Drosophila* Adult Eye Development with Other Holometabolous Species: Early Versus Late Eye Discs

The physical separation of the products of embryonic and postembryonic eye development in holometabolous species dominates the comparison of *Drosophila* to direct-developing insect species (Fig. 2). The comparison of eye development within holometabolous species attracts interest because of the dramatic differences in the morphogenetic organization of postembryonic eye primordium formation (Fig. 9). In the most ancestrally organized Holometabola, the retina differentiates in

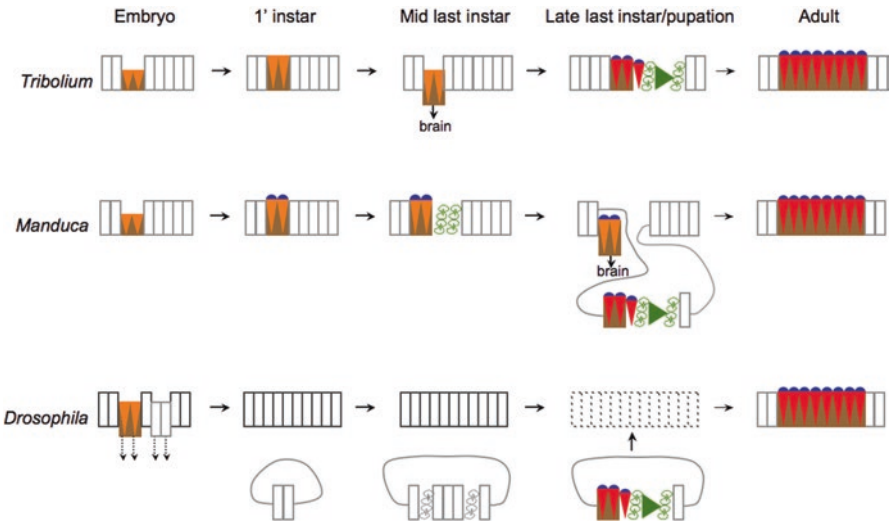

Fig. 9 Early and late eye disc formation in holometabolous insects. Cell body color coding as in Fig. 3. Note the differentiation of photoreceptors with cone cells in the tobacco hornworm *Manduca sexta*. In *Tribolium*, the adult retina differentiates in the lateral head epidermis without eye disc formation. In *Manduca*, a later eye disc is formed in the last larval instar and the pupa. The *Drosophila* eye-antennal imaginal disc is an example of early imaginal disc formation in the embryo

the lateral epidermis of the adult-like head capsule of the eucephalic larva. Pending the size of the prospective adult eye, this can be associated with the formation of an eye disc during metamorphosis, i.e., the last larval instar and the pupa. This contrasts with the early formation of the *Drosophila* eye-antennal imaginal disc during embryogenesis (Friedrich 2006).

Correlated with this, there is a second fundamental morphogenetic difference between the ancestral late, i.e., postembryonic, formation of eye disc and the early eye disc development in *Drosophila*. In the first case, the eye disc is the growth-accommodating intermediate structure of a single organ. In the second case, the eye-antennal imaginal disc functions as the precursor of many head cuticle structures and sensory organs (see also Fig. 15). This has the effect that organ-specific primordia have to be patterned via postembryonic regional specification in addition to their coordinated growth (for review see Dominguez and Casares 2005). This compaction of head patterning processes into a single composite imaginal disc represents a derived state that emerged during the evolution of the acephalic morphology of the maggot-type larva (Melzer and Paulus 1989). The latter characterizes not only *Drosophila* and closely related flies but one of the larger groups of the Diptera: the Cyclorrhapha. The early eye disc of *Drosophila* and other cyclorrhaphan flies thus represents an evolutionary novelty at the level of developmental precursor tissue organization.

The Red Flour Beetle *Tribolium castaneum*

The publication of its genome sequence in 2008 cemented the pivotal position of *Tribolium* in comparative evolutionary developmental biology (Klingler 2004; Richards et al. 2008). The recent surge in *Tribolium* research benefited from early genetic and population genetic studies exploring the biology of this major economic pest (Sokoloff 1972). The comparative significance of *Tribolium* is due to representing the largest order of insects (Coleoptera) and the intermediate phylogenetic position between *Drosophila* and hemimetabolous insects (Fig. 1) (Kristensen 1999; Savard et al. 2006; Wiegmann et al. 2009). These aspects and the short germ band type of embryonic development have attracted considerable interest, leading to the development of refined and effective protocols for in situ hybridization, RNAi-mediated gene knockdown, transgenesis (Brown et al. 2009), and ectopic gene expression (Schinko et al. 2012). *Tribolium* has been used to gain insights into early embryonic patterning (Schroder 2003), segmentation (Maderspacher et al. 1998), appendage (Prpic et al. 2001), and head development (Posnien et al. 2010), including the visual system (Liu and Friedrich 2004).

Organization of the *Tribolium* Compound Eye

A first notable difference of the *Tribolium* eye to *Drosophila* is its smaller size: an average of 95 ommatidia in the *Tribolium* eye compares to the 800 ommatidia in the *Drosophila* eye (Fig. 10f) (Friedrich et al. 1996). This size difference can be attributed to the crepuscular biology of *Tribolium*, which tends to spend much of its life span borrowed in nutritional substrate interrupted by flight-facilitated adult dispersal (Perez-Mendoza et al. 2011; Ridley et al. 2011; Park 1934).

A second eye-catching difference between the *Tribolium* and *Drosophila* eye is the midline notch at the anterior margin of the *Tribolium* eye, accommodating a posteriorly extended gena (Fig. 10e and f).

At the cellular level, the fused rhabdom formed by *Tribolium* photoreceptor cells contrasts with the open rhabdom in *Drosophila* (Friedrich et al. 1996). Only two opsin paralogs are expressed in the *Tribolium* retina (Richards et al. 2008): a green-sensitive opsin, which is expressed in all retinal photoreceptor cells, and a UV-sensitive opsin, which is specifically expressed in the *Tribolium* R7 photoreceptors (Jackowska et al. 2007). In combination, the *Tribolium* retina thus differs from *Drosophila* by the constitutive co-expression of opsin paralogs in all ommatidia. Recent studies defined the contributions of the transcription factors *glass, orthodenticle* 1, *orthodenticle* 2, and *PvuII-PstI homology* 13 (*Pph13*) in the differential activation of opsin gene expression in the *Tribolium* photoreceptors (Friedrich et al. 2016; Liang et al. 2016; Mahato et al. 2014).

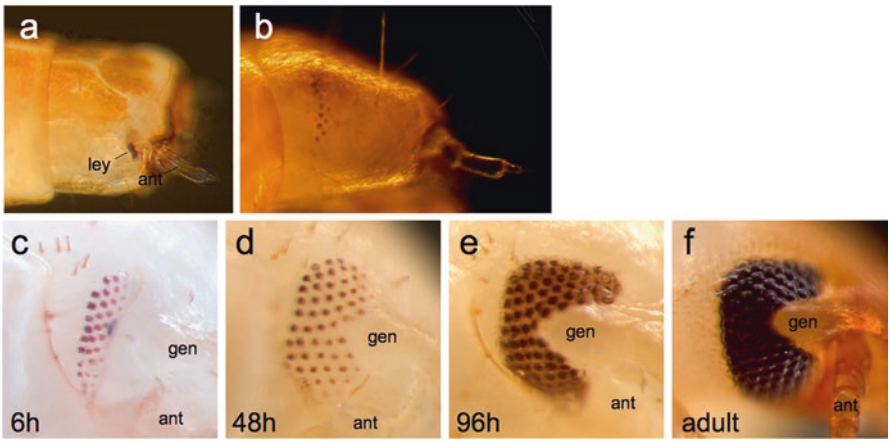

Fig. 10 Adult eye development in *Tribolium*. (**a**) Lateral view of last instar larval head before entering the resting stage. Note position of larval eyes (ley) posterior to the antenna (ant). (**b**) Lateral view of resting stage larva. The larval eyes have relocated from their antenna-associated position toward the brain (not shown). The first two rows of photoreceptors, visible by virtue of their pigment accumulation, have become visible in the posterior half of the lateral head capsule. (**c**–**f**) Lateral view of pupal (**c**–**e**) and freshly hatched adult (**f**) *Tribolium* head. Adapted from Liu and Friedrich (2004) and Yang et al. (2009b)

Morphogenesis of the *Tribolium* Compound Eye

Like *Drosophila*, *Tribolium* develops larval eyes in the embryo that are structurally very distinct from the adult compound eye. The larval eyes are situated close to the larval antenna from where they withdraw into the brain during metamorphosis (Fig. 10a and b) (Liu and Friedrich 2004; Friedrich 2013). The relative small size of the adult *Tribolium* eye allows for the differentiation of the retina in the lateral head epithelium without detaching from the head cuticle (Figs. 9 and 10). Due to the early accumulation of retinal pigment granules in differentiating photoreceptor cells, the morphogenesis of the *Tribolium* compound eye can be conveniently followed by external observation (Fig. 9) (Friedrich et al. 1996; Liu and Friedrich 2004). The first row of photoreceptors is recognizable at the end of the last larval instar (Fig. 10b), while in preparation for pupation. At this point, the larvae enter a similar pre-metamorphic stage that as the wandering stage of the larva of *Drosophila*. In the case of *Tribolium*, however, the larvae simply remain motionless without food uptake (Parthasarathy et al. 2008).

In the freshly hatched pupa, the number of photoreceptor columns extends in anterior direction over the first 48 h after pupa formation (Fig. 10c and d) (Liu and Friedrich 2004; Yang et al. 2009b). In the midline area, the progression of photoreceptor differentiation stalls earlier than in the dorsal and ventral halves (Fig. 10d and e). Investigations of cellular morphogenesis revealed that this process is associated with the split of the initially contiguous morphogenetic furrow in the midline region (Friedrich and Benzer 2000). About 96 h after pupa formation, the retinal

field becomes homogeneously filled with pigment following the specification and differentiation of the pigment cells (Yang et al. 2009b).

Signaling Factor Expression Patterns in the Developing *Tribolium* Compound Eye Retina

The first molecular study of *Tribolium* eye development explored the expression patterns of *wg* and *dpp* (Fig. 11) (Friedrich and Benzer 2000). Similar to the situation in grasshopper and *Drosophila*, *wg* is expressed in separate dorsal and ventral domains, consistent with evolutionary conservation of the repressive effect of Wg signaling on retinal differentiation in *Drosophila* and the grasshopper (Dong and Friedrich 2005).

During pupal development, these dorsoventral *wg* domains transform into a circumferential domain along the entire retinal field margin, resembling the late expression of *wg* around the *Drosophila* eye (Friedrich and Benzer 2000). The expression of *dpp* in *Tribolium*, however, is different from both grasshopper and *Drosophila* (Friedrich and Benzer 2000). At the onset of retinal differentiation, *dpp* is weakly expressed in the presumptive eye primordium (Fig. 11). After the

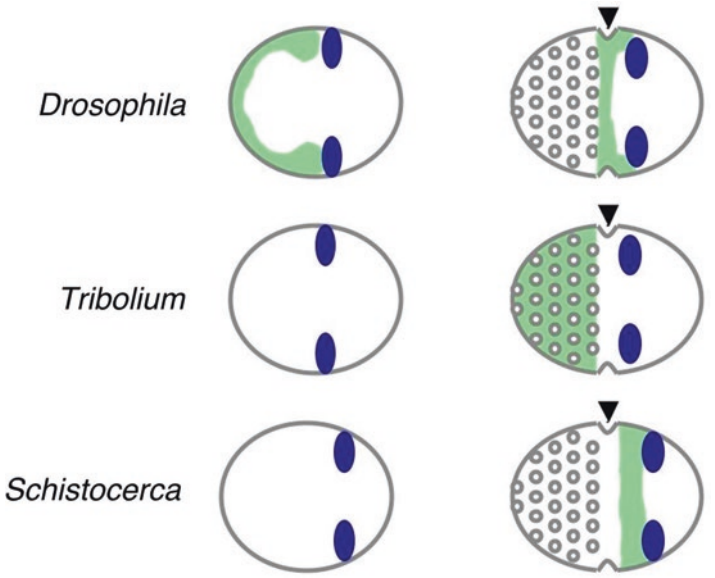

Fig. 11 Comparison of *wg* and *dpp* expression domains in *Drosophila*, *Tribolium*, and *Schistocerca*. Left column represents the eye field before the onset of retinal differentiation. The right column represents the eye field after the onset of retinal differentiation. Arrowheads point at the front of retina differentiation. Color code of gene expression domains: *dpp* = green, *wg* = blue; Modified from Friedrich and Benzer (2000)

initiation of retinal differentiation, *dpp* appears weakly expressed in the differentiating retina in a pattern, which suggests repression in the emerging photoreceptor cells.

Eye Selector Gene Expression in the Developing *Tribolium* Compound Eye Retina

Also the expression of *eya*, *so*, *dac*, and the *Pax6* transcription factor genes *eyeless* (*ey*) and *twin of eyeless* (*toy*) has been studied in the developing *Tribolium* eye (Figs. 12 and 13) (Yang et al. 2009a, b). All of these genes are expressed in the undifferentiated eye primordium prior to retinal differentiation and subsequent to the initiation of differentiation ahead of the morphogenetic furrow, suggesting their co-expression in the early eye primordium (Fig. 12a–c). The extent of these expression domains, however, differs. *Eya* and *so* appear to be more specifically expressed in the retinal precursor tissue of the lateral head (Fig. 12c). *Ey*, *toy*, and *dac,* by contrast, are characterized by wider expression domains, exceeding that of *so* and *eya*, suggesting broader roles in the patterning of the lateral head (Fig. 12a and b) (Yang et al. 2009a).

Informative expression pattern differences were also observed in the differentiating retina. While *eya* and *so* continue to be expressed in the developing photoreceptor cells, *ey*, *toy*, and *dac* are downregulated as cells pass through the morphogenetic furrow. These expression dynamics are largely consistent with the expression and function of *eya* and *so* as early retina determination genes versus *toy* and *ey* as

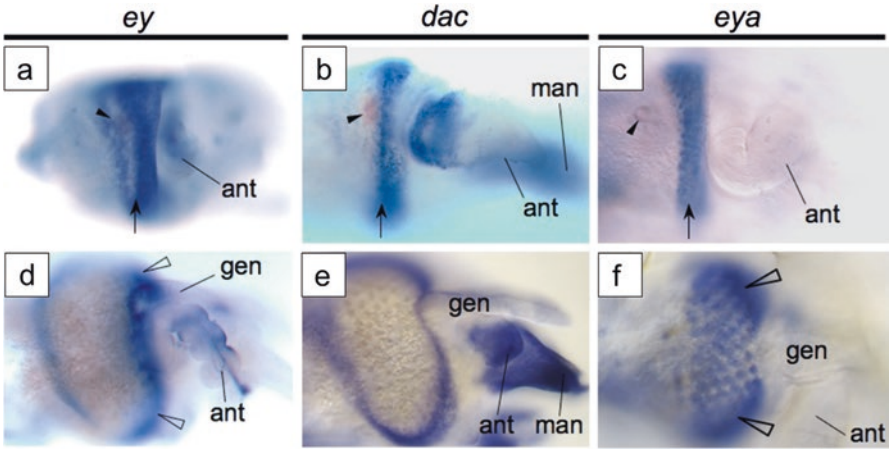

Fig. 12 Developmental transcription factor gene expression in the developing *Tribolium* compound eye. (**a**–**c**) Lateral view of dissected last instar larval head. (**d**–**f**) Lateral view of pupal head at approximately 48 h after pupal formation. Dorsal up and anterior to the right. Abbreviations: ant = antenna, gen = gena, man = mandible

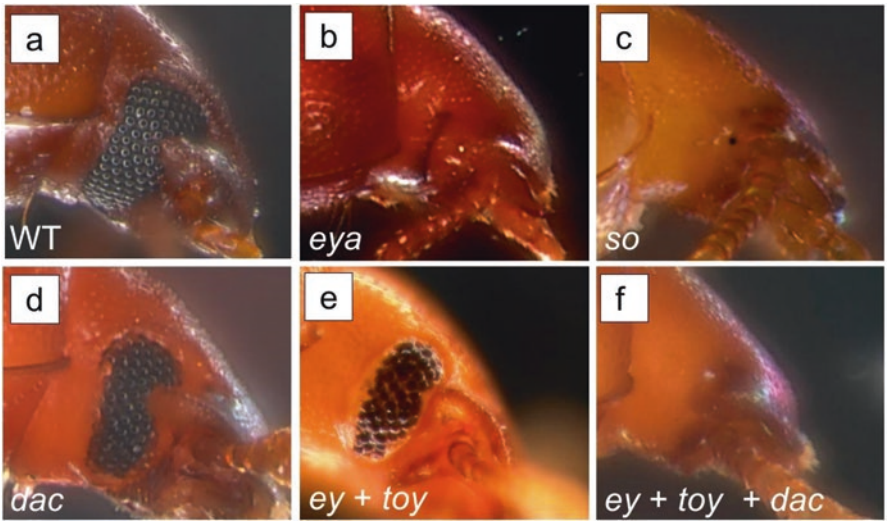

Fig. 13 Eye selector gene expression and function in *Tribolium* compound eye development. (**a**–**f**) Lateral view of adult head of (**a**) wild-type and (**b**–**f**) strongly phenotypic knockdown animals. See text for details. Adapted from Yang et al. (2009a, b). Dorsal up and anterior to the right

upstream specification genes in the *Drosophila* eye-antennal disc (Kumar 2009) (Atkins et al. 2013). Most noteworthy perhaps is the coordination of *dac* expression with *ey* and *toy* in *Tribolium* (Fig. 12d and e), considering the downstream position of *dac* in the *Drosophila* retina determination gene network. Further emphasizing this notion, these three genes are also co-expressed in a domain surrounding the late differentiating *Tribolium* retina suggesting roles in eye margin patterning (Fig. 12d and e) (Yang et al. 2009a, b). Analysis of the roles of *ey*, *toy*, and *dac* during embryonic development revealed that these genes are essential for the early specification of the ocular segment, i.e., the compartment of the developing head from which both the larval and ultimately the adult visual system develop (Luan et al. 2014).

Knockdown Analysis of Eye Selector Gene Function in *Tribolium*

The roles of *eya*, *so*, *ey*, *toy*, and *dac* in the early development of the *Tribolium* compound eye have been subjected to extensive gene knockdown investigations. The RNAi-mediated gene knockdown of *eya* and *so* triggered partial to complete depletion of the compound eye (Fig. 13b and c) (Yang et al. 2009b), consistent with the requirement of both genes for visual development in *Drosophila* (Bonini et al. 1993; Daniel et al. 1999; Pignoni et al. 1997). The analysis of *ey* and *toy*, however, pointed at major differences in their gene interactions in the eye determination gene network between *Tribolium* and *Drosophila*. Knocking down *ey* or *toy* individually or in

combination leads to only a subtle decrease in eye size as measured by number of ommatidia (Fig. 13e) (Yang et al. 2009a), contrasting with the sensitivity of adult head and eye development to the reduction of these genes in *Drosophila* (Kronhamn et al. 2002).

The knockdown of *dac* also yielded only partial reduction of the eye (Yang et al. 2009a). Most important, the combinatorial knockdown of *ey*, *toy*, with *dac* leads to complete eye depletion (Fig. 13f) (Yang et al. 2009a). The model inferred from these data is that *ey* and *toy* are essential for eye primordium maintenance in functional redundancy with *dac* (Yang et al. 2009a).

An Unexpected Role of *eyg* in the *Tribolium* Eye

A second major difference in the genetic control of eye development between *Tribolium* and *Drosophila* concerns the role of the Pax gene *eyegone* (*eyg*) (ZarinKamar et al. 2011). Reducing *eyg* levels in the *Drosophila* eye-antennal disc has strong eye depletion effects (Dominguez et al. 2004; Jun et al. 1998). In *Tribolium*, however, the knockdown of *eyg* leads to the opposite: a 5% increase in eye size (ZarinKamar et al. 2011). Analysis of the morphogenetic origin of the *eyg* phenotype in *Tribolium* revealed that the morphogenetic furrow is not suppressed in the midline when approaching the introducing gena tissue. In this case, retinal differentiation in the median head appears to gain dominance over the developmental program involved in gena formation. The result is the differentiation of on average six surplus ommatidia in the median anterior *Tribolium* eye (ZarinKamar et al. 2011). Given that *eyg* is not expressed in the developing gena itself, it has been suggested that *eyg* functions as a competence factor that renders the anterior eye field sensitive to retina suppressing factors released by the developing gena (ZarinKamar et al. 2011).

The Tobacco Hornworm *Manduca sexta*

Compared to *Tribolium*, the tobacco hornworm *M. sexta* has played a lesser role in the comparative analysis of visual system development. Early work described basic aspects of the differentiation of its large compound eye retina, which align well with the events in the wake of the morphogenetic furrow in *Drosophila* (Champlin and Truman 1998; Egelhaaf 1988; Friedrich et al. 1996). Even more significant is the body of work, which elucidated the mechanisms that regulate the postembryonic activation of adult eye primordium (Champlin and Truman 1998; Truman et al. 2006). These experiments revealed that the initiation of adult eye primordium growth and differentiation is mediated by nutritional signals through the insulin signaling pathway which relieve the differentiation-suppressing effect of juvenile hormone (Koyama et al. 2008; Truman et al. 2006).

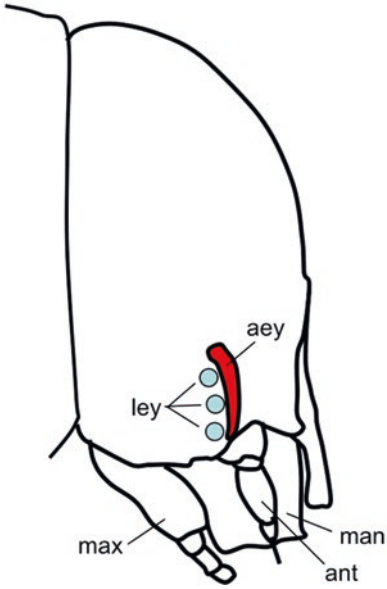

Fig. 14 Spatial organization of adult eye primordium initiation in relation to the larval eyes in *Manduca*. Drawing of lateral view on *Manduca* final instar larval head based on Allee et al. (2006). The adult eye primordium is initiated as a wedge of proliferating tissue anterior to the three ommatidia-like larval eyes (turquoise). Dorsal is up and anterior to the right. Abbreviations: aey = adult eye primordium, ant = antenna, ley = larval eye, man = mandible, max = maxilla

Early Development of the *Manduca* Compound Eye Primordium

As mentioned above (Fig. 9), *Manduca* is a significant point of comparison in insect eye development because of the late formation of an eye-specific imaginal disc (Allee et al. 2006; Friedrich 2006; Truman and Riddiford 2002). The adult eye primordium of *Manduca* becomes detectable in the late final instar larva. Morphologically, it has been described as a rim of compact, proliferating tissue that delaminates from the larval head capsule cuticle (Fig. 14) (Allee et al. 2006; MacWhinnie et al. 2005; Monsma and Booker 1996). This position of the emerging eye disc is notable because it is consistent with the transient arrest model of the larval eye evolution in holometabolous insects. The latter predicts that the adult eye primordium is initiated as a continuation of larval eye development and thus at the anterior margin of the larval eye (Fig. 2).

Eye Specification Across Insect Species: Summary and Perspectives

From both a phylogenetic and developmental point of view, the diversity of adult eye morphogenesis is enormous in insects, posing challenges to the experienced comparative biologist and the weathered *Drosophila* geneticist alike. Notwithstanding this, some of the available molecular data define ancestral

themes in the early development of the compound eye in both direct- and indirect-developing species.

Conserved Involvement of Eye Selector Genes

Arguably the clearest example of this is the involvement of *eya* and *so* as facilitators of retinal precursor tissue determination and subsequent retinal differentiation (Figs. 8 and 12). A similar point may be made regarding *dac*, *ey*, and *toy*. These genes share broad expression patterns that include the retinal precursor tissue and are downregulated in the differentiating retina, pointing at a conserved role in implementing competence for retinal determination (Fig. 12). Taken together, these data are consistent with the roles experimentally ascribed to *eya*, *so*, *dac*, *ey*, and *toy* in *Drosophila* (Kumar 2009). The conserved expression of *eya* and *so* is further suggestive of a broad conservation of the PPN state of retinal commitment, at least at the transcription factor landscape level (Bessa et al. 2002; Dong and Friedrich 2005; Greenwood and Struhl 1999).

Conserved Interplay of Signaling Factor Input

At the signaling factor level, the repressive effect of *wg* in the anterior developing eye field is a similarly highly conserved aspect of insect compound eye development. This is reflected in the conservation of polar expression domains in the anterior eye precursor field of all insect species so far examined (Fig. 5) and even in a crustacean species (Duman-Scheel et al. 2002). Although the expression patterns of *dpp* are quite diversified in the developing eyes of different species (Fig. 5), the eye development-promoting role of *dpp* can likewise be presumed to be conserved. The same applies to the retinal differentiation-promoting role of *hh*, based on the data from crickets at this point (Niwa et al. 2000).

Divergence of Eye Primordium Growth Activation

The findings in the comparative analysis of *eyg* suggest the possibility of profound differences between *Drosophila* and more ancestrally organized insects when it comes to the activation of tissue growth in the compound eye primordium. In *Drosophila*, *eyg* is part of the N-signaling-induced growth-promoting genetic network that is pivotal for triggering the rapid tissue growth in the developing eye disc (for review see Dominguez and Casares 2005). The discrepancy of *eyg* function in *Tribolium* and *Drosophila* may thus be explained by the smaller size of the eye in *Tribolium*, requiring less tissue proliferation. A second possibility is that the

N-signaling-mediated organizer originated more recently in conjunction with the evolution of the *Drosophila* eye disc during dipteran evolution (Melzer and Paulus 1989). Of note, an evolutionarily derived status of the N-initiated growth activation mechanism on *Drosophila* and related Diptera would also explain the non-compartmentalized expression patterns of *fng* and *Dl* in grasshopper (Dong and Friedrich 2005). A new data point in support of this model has come from the silk moth. Similar to *Manduca,* this lepidopteran develops its 3,000 ommatidia large compound eye from a late forming eye disc (Yu et al. 2012). The silk moth mutant *flügellos* has been found to represent a null allele of *Bombyx* homolog of *fng* (Sato et al. 2008). Importantly, while *flügellos Bombyx* animals are characterized by wing defects, the development of the compound eye is not affected in dramatic ways. This suggests that the dramatic growth of the lepidopteran eye does not depend on *fng* as in *Drosophila*. In conclusion, the N- and *eyg*-dependent activation of growth in the *Drosophila* eye disc may not be a conserved component of eye disc development in holometabolous insects.

Embryonic Versus Postembryonic Determination of the Adult Eye Primordium

Another fundamental question still waiting to be addressed concerns the specification of the adult retina primordium in ancestrally organized holometabolous species like *Tribolium* and *Manduca*. To get a taste of the foundational nature of this issue, one has to remember that the late postembryonic specification of the adult eye primordium in *Drosophila,* based on molecular genetic analysis, came as a surprise to the *Drosophila* field (Baker 2001; Kumar and Moses 2001). The preceding consensus was that this step takes already place in the embryo, during the subdivision of the embryonic visual anlage into its major constituents (Postlethwait and Schneiderman 1971; Wieschaus and Gehring 1976). Assuming that the late specification of the eye primordium is the consequence of the evolution of the highly derived eye-antennal imaginal disc of *Drosophila* (Fig. 13), it is reasonable to hypothesize that the specification of the adult eye primordium does take place during embryogenesis in species with late eye discs like *Manduca* or no disc formation like *Tribolium* (Fig. 9). Otherwise, one has to postulate a postembryonic patterning mechanism, which drives the specification and activation of the adult eye primordium in the lateral head epithelium of the last instar larva.

Also the comparative framework of the transient arrest model of holometabolous visual system development predicts that both larval eye and adult eye precursor cell populations are committed in the embryonic visual anlage (Fig. 2). In the embryo, differentiation is initiated in the larval eye precursor cells but suppressed in the adult eye precursor cells. The latter, embedded in the lateral head epidermis, are maintained as a quiescent primordium until activation at the beginning of

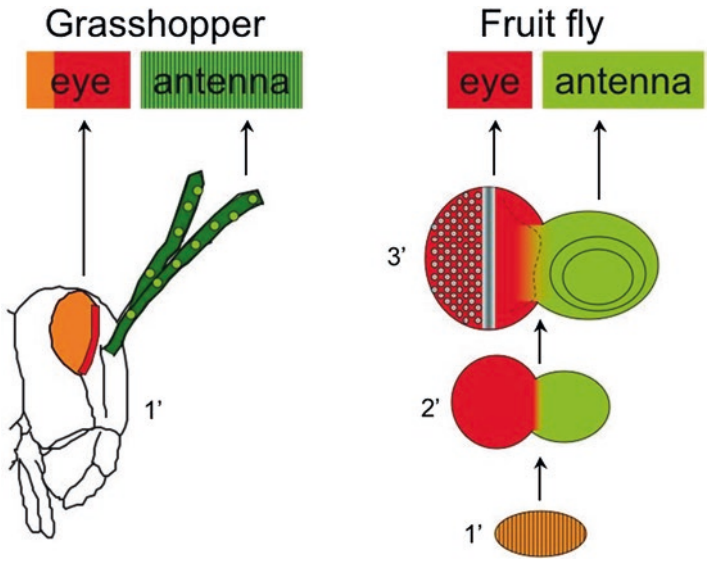

Fig. 15 Somatic stem cell reservoirs versus imaginal discs in insect eye development. In direct-developing insects like grasshopper, the adult antenna and compound eye derive from organ-specific stem cell reservoirs (eye, red; antenna, light green) and differentiated cells of the nymph (eye, orange; antenna, dark green), which have been generated during embryogenesis. This mode of organ precursor tissue organization contrasts with the development of adult antenna and compound eye from the joint eye-antennal imaginal disc of *Drosophila*, which undergoes dramatic morphogenetic change through all three larval instars (1′–3′)

metamorphosis. This scenario is consistent with the positioning of the adult eye primordium in front of the larval eye in *Manduca* (Allee et al. 2006).

Important work remains to be done to elucidate whether and how the precursor cells of the adult eye are set aside during embryonic development in more ancestrally organized systems like *Tribolium* and *Manduca* (Fig. 9). While interesting in its own right, answers to these questions will also yield insights of broader significance. For one, they will add to our understanding of the molecular developmental evolution of holometabolous development, which after all was co-responsible for the unparalleled radiation of holometabolous insects (Kristensen 1999). Furthermore, the comparative evidence implies that the *Drosophila* eye-antennal imaginal disc is a derivative of the retinal growth zone in direct-developing insects, which most likely represents a tissue-specific stem cell population (Dong and Friedrich 2010). If confirmed, the evolutionary transformation of the retinal growth zone in directly developing species to the *Drosophila* eye-antennal imaginal disc would be an example of how evolution reprogrammed stem cell populations to invent novel ways of body plan development (Fig. 15).

Acknowledgments We are grateful to Amit Singh for the kind invitation to provide a revised and updated version of this book chapter.

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Eye for an Eye: A Comparative Account on Compound Eye of *Drosophila melanogaster* with Vertebrate Eye

Arushi Rai, Sonia Narwal, Harsh Kanodia, and Meghana Tare

Introduction

Eyes, as mentioned by philosopher William Paley, are "miracle of design." Eyes are indeed amazing organs in the animal kingdom, for their ability to provide a unique sense that makes most of the animals stand apart from rest of the living organisms. Although not all kingdoms of life are devoid of visual senses, the ability to connect sense of vision to that of complex nervous system for processing and image formation is unique to the animal kingdom. Diversity of the eyes in the animal kingdom has been attributed to evolution over a large period of time. Based on evidences from fossil records, first eyes appear some 540 million years ago (Parker 2009). There are different kinds of eyes animals possess, which work in different fashions, in order to "sense" the objects, and may be to form an image. Of all diverse life forms, eye of *Drosophila melanogaster* is an example of *eyes; for an eye*; for, it has compound eyes, for sensing, processing and forming the image. For over a century now, *Drosophila melanogaster* eye has provided a new dimension to several different aspects of understanding in the fields of development and several different diseases (Borst 2009). Santiago Ramon y Cajal, a neuroanatomist was the first to notice the similarities between the visual system of vertebrates and that of the insects. He documented a striking similarity between the neuronal circuits that form the major framework of visual system in flies and vertebrates (Cajal and Sanchez 1915). Compound eyes are built as convex structures around the outside of an animal's head, and even though their arrangement looks similar to vertebrate eyes (both sides of head), they are fundamentally different from the concave structure of single chamber eyes (Fig. 1). In spite of this major topological difference, however, the

A. Rai · S. Narwal · H. Kanodia · M. Tare (✉)
Department of Biological Sciences, Birla Institute of Technology and Science, Pilani, Pilani, Rajasthan, India
e-mail: meghana.tare@pilani-bits.pilani.ac.in

A. Singh, M. Kango-Singh (eds.), *Molecular Genetics of Axial Patterning, Growth and Disease in Drosophila Eye*,
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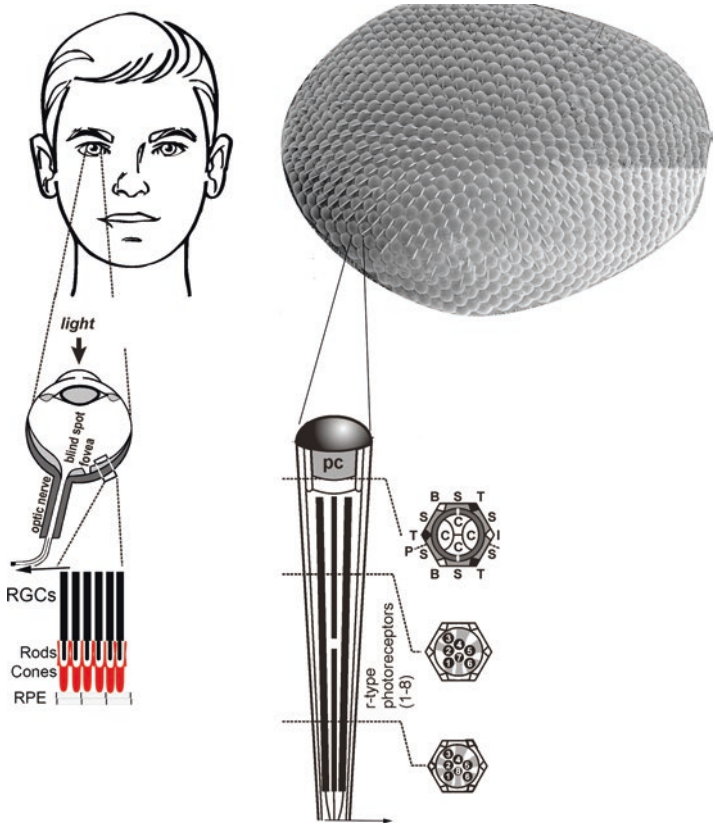

Fig. 1 A vertebrate eye versus *Drosophila melanogaster* eye. Vertebrates have single camera type eyes compared to compound eyes of *Drosophila*. (**a**) In the vertebrate eye, light rays falling are refracted by the cornea (outer protrusion) and lens (oval structure inside) onto PRCs in the neural retina. Cellular arrangement for Retinal Ganglion Cells (RGCs), and rods and cones has been shown in the enlarged portion of the eye. Arrow marks the direction of axons to CNS. (**b**) Compound eye of *Drosophila* is made up of regularly placed facet like structures, each referred to as ommatidium. Each ommatidium appears like a cylindrical structure tapered at the end. Pseudocone (PC), of each ommatidium is secreted by cone cells (C in the section). Eight of the R-type photoreceptor cells (PRCs), labeled as R1–R8. R1–R6 span across the height of the ommatidium. R7 and R8 lie above and beneath the hexagon. Primary (P), Secondary (S) and Tertiary (T) pigment cells encase the photoreceptor cells and function in absorbing wondering photons. At regular intervals, Bristle (B) cells replace the T cells. Grey areas in the cross sections represent the five of the opsins in image formation. Arrow marks the direction of axons to CNS. (Image adapted from Lewis Held 2017)

jobs of the two kinds of structure are the same: to utilize the incoming light and to develop a sense of vision (Pak 2010; Sanes and Zipursky 2010). For eye is of interest to many research fields, in order to stay focused, we compare the anatomy and function while dwelling into events of genesis of the eye in the embryonic stages, and their genetic regulation.

We shall provide the major similarities and differences in the structure, function, and development of the camera type eyes with those of compound eyes of *Drosophila melanogaster* in subsequent sections.

Anatomy of Vertebrate Eye

The arrangement of the eye is extremely intricate as indicated (Fig. 1). The entry of light into the eye is facilitated by the cornea. The cornea is thin and transparent. Its transparency arises from an acellular stroma between a layer of epithelial cells and a layer of endothelial cells. It contains no blood vessels to avoid attenuating the light entering the eyes. The cornea receives nourishment from tears on the outside and aqueous humor on its inner surface. The cornea acting in conjugation with the lens focuses light onto the light detecting cells of the eyes—the photoreceptors. The lens too is highly transparent, an adaptation to maximize the light transmitted into the light-sensitive cells of the eye. The lens allows for its shape to be changed in order to allow accommodation of images at different distances and change the focus of the lens. The lens is held in place by the zonular fibers that extend to ciliary body. The contraction of the ciliary muscles facilitates the change of shape of the lens. The forces of ciliary muscles are conveyed to the lens via the zonular fibers. The contraction of the ciliary muscles releases the tension in the zonular fibers and allows the lens to become more round allowing change in the focal plane of the lens-cornea system. Though the cornea achieves most of the focusing function, it has a fixed focus, thus imparting the important function of accommodation to the lens. The lens unlike the cornea is transparent due to the nature of lens cells that constitute it.

The lens fiber cells lose their nuclei and most of their organelles during differentiation. They have high content of proteins called crystallins which do not scatter light like most other proteins. The crystallins have interestingly shown to be expressed in other cells in the body where they have different functional roles such as enzymatic activity (Piatigorsky and Wistow 1989). The iris regulates the entry of light in through the lens. It can dilate or constrict its opening, thus attenuating the light to different extents. The space anterior to the lens is filled with a fluid known as the aqueous humor which is responsible for maintaining the pressure in this compartment of the eye and gives it its shape. The ciliary bodies secrete the aqueous humor. The aqueous humor leaves the eyes through tiny channels in the periphery of the anterior chamber. Posterior to the lens is the vitreous humor which is a denser fluid gel. It exerts a pressure that keeps in place the retina—which is the neuron rich layer responsible for visual computations and relaying the information regarding the visual field to the higher centers in the brain. The retina is followed by the pigmented epithelium and they line the posterior end of the eye. They are followed by the choroid which is rich in vasculature and supplies the outer retinal cells and the photoreceptors together with the pigmented epithelium with nutrients and facilitates gaseous exchange. The output neurons of the retina project to the brain regions via the optic nerve, which is composed of the axons, called the retinal ganglion cells

(RGCs) of the retina (the output neurons). The outermost coat of the eye is a tough layer known as the sclera, which is a white tissue. The inner retinal cells receive nourishment and gaseous exchange via the repeated branching of retinal artery.

After portraying the anatomical organization of the eye, it becomes important to understand the retina—the most important part for the early processing of the visual scene and encoding it to be processed by higher brain regions. The retina has a vast diversity in constituent cell types (Fig. 1) that all play a role in the computations performed by the retina that maybe categorized on the basis of molecular identity, morphology, and dendritic stratification patterns (Baden et al. 2016; Gollisch and Meister 2010; Masland 2001, 2012). The subtypes of each cell show a regular arrangement—*i.e*., there exists a region of exclusion around each cell, where other cells of the same subtype are not found. This leads to a mosaic-like arrangement of each non-reducible neuronal cell subtype—a characteristic feature of the retina. These cells help to convert the image perceived in the visual field into parallel streams of information regarding various features of the image. The neurons of the retina are organized in three cellular layers—the ganglion cell layer, the inner nucleate layer, and the outer nucleate layer. There are two synaptic layers—the inner and outer plexiform layers. These synaptic layers show further stratification. There are six major cell types in the vertebrate retina—the photoreceptors, the horizontal cells, the bipolar cells, the amacrine cells, the ganglion cells, and the glial Muller cells. The photoreceptors—rods and cones—receive photostimulation due to the photopigments (opsins) in these cells responding to impinging photons. The opsin proteins are bound to retinal—a form of Vitamin A. The molecule undergoes isomerization upon absorption of photons, the photosensitive reaction that drives a signaling cascade underlying the function of the retina. The photoreceptors project to the outer nucleate layer where they synapse with the horizontal cells and bipolar cells. The photoreceptors use glutamate as a neurotransmitter. Upon impingement by light, the photoreceptors hyperpolarize—their membrane potential decreases. This leads to a reduced secretion of glutamate which effects the bipolar cells and horizontal cells downstream. The bipolar cells show different functional responses to the light responses of the photoreceptors based on the type of glutamate receptors (both ionic and metabotropic) they express—for example, ON bipolar cells express metabotropic mGluR6 which causes reduced depolarization of the bipolar cell membrane upon binding the glutamate, and hence, when light causes lowered glutamate release from the photoreceptor cells, these cells show increased depolarization of membrane and an ON response to increase in light intensity in their receptive fields. The horizontal cells play a role in feedback and modulate the responses of the photoreceptors. The bipolar cells show wide diversity (Tsukamoto and Omi 2013). The bipolar cells then contact ganglion cells in the inner plexiform layer. Here, a divergence of information occurs and various arrangements of these synaptic contacts and interaction and modulation by the amacrine cells allow for a variety of computations. The ganglion cells have over 30–40 types (Baden et al. 2016) and carry parallel information to the brain about the visual scene. The complex interplay of signals from the bipolar, amacrine, and retinal ganglion cells plays an important role in various features detected and encoded by the retinal ganglion cells. Some

instances of these computations include object motion (Baccus et al. 2008), approaching motions (Münch et al. 2009), motion extrapolation amongst other forms of anticipation and adaptations (Chaffiol et al. 2017; Gollisch and Meister 2010; Yao et al. 2018). There are a wide variety of neurotransmitters and receptors involved and they have been implicated in a variety of different functional computations—for instance, dopamine has been implicated in light adaptation of the retina, where the retinal dopamine levels go up with increase in light intensity and seem to be involved in a variety of light adaptive computations that may not be explained by a simple gain control of the retinal cells (Chaffiol et al. 2017; Yao et al. 2018). At the same time, a number of adaptions and functionality of the retina depend on inputs from the brain—retinopetal inputs. This makes it interesting to look at the modulation of signals by various neurotransmitters which are released into the retina by retinopetal neurons in a context-dependent manner. Thus, the mechanism by which the retina computes information cannot be studied independent of these modulating signals.

Anatomy of Drosophila *Eye*

The major structural components in the retina of *Drosophila* are the 750 individual units termed as ommatidia which are precisely organized in the lattice (Fig. 1). Each ommatidium consists of eight R cells which are basically the photoreceptor neurons (R1–R8). The photoreceptors can be categorized it is on the basis of opsins they express: R1–R6 type of photoreceptors expresses Rh1 opsins and controls the motion detection, secondly R7 expresses RH3 or Rh4 opsins which are UV-sensitive and lastly R8 expresses either Rh5 (blue) or Rh6 (green) opsins (Salcedo et al. 1999). The photoreceptor cells direct its visual information towards the **optic lobe**, the primary visual processing center in flies. This optic lobe is composed of four ganglia. First layer is called lamina, beneath it lays the medulla and then the lobula. Mainly in flies, the lobula is further differentiated into lobula and lobula plate (Sinakevitch et al. 2003). The R1–R6 photoreceptors terminate in the first layer lamina while the axons of R7 and R8 end at medulla and hence medulla receives information from the either R7 or R8. In both the R7 and R8 cells, a zinc finger transcription factor called as Sequoia and some N-cadherins are expressed but they majorly control the precise positioning of the axons of photoreceptor R7. Another cell adhesion molecule called Capricious is expressed selectively in R8 cells and regulates the projection of axons of R8 cells (Kulkarni et al. 2016).

The neural circuits are formed of four types of neuronal cells, local neurons or intrinsic neurons, interneurons, photoreceptor axons, and visual projection neurons (VPNs). VPNs connect the optic lobe and the central brain, intrinsic neurons ramify within a single optic ganglion, and interneurons connect more than one ganglion within the optic lobe. Intrinsic neurons, interneurons, and the axons of photoreceptors are oriented in a parallel direction creating a barrel-like structure called the

visual cartridge (Otsuna and Ito 2006). The photoreceptor cells collect information from different point and converge it into these parallel columnar synaptic models. The axon of R1–R6 terminates in the lamina and further directs the motion information to the neurons of lamina (L1–L5) in synaptic units. These synaptic units along with amacrine cells and centrifugal interneurons are termed as laminal cartridge (Meinertzhagen and O'neil 1991). The motion information is further transmitted to the underneath ganglia medulla through the axons of lamina neurons L1–L5 each arborized in the particular medulla layers. Along with the axonal projection of the laminal neurons, the axons of R7 and R8 transmit the color information to the M6 and M3 medulla layers, respectively (Takemura et al. 2008; Morante and Desplan 2008). Hence, the parallel columnar organization of the 750 lamina cartridges and medulla column relays the information in a retinotopic fashion that allows the parallel processing of the visual information from different points.

The fly visual system is made up of different neuronal cell types based on the morphology. It can mainly be categorized into two main classes: the uni-columnar neurons and multi-columnar neurons. The uni-columnar neurons are mainly restricted to one column and its projections extend laterally connecting the neighboring columnar modules. The multi-columnar neurons project in several columnar modules. This parallel relay of information either between the layers or columns optimize the signal-to-noise ratio.

Phototransduction and Image Formation

Compound eyes are apposition kind of eyes where optically isolated ommatidia process the images separately. Apposition eyes are typically optimized for high resolution by "apposing" little overlapping visual fields of neighboring ommatidia based on small apertures and rhabdoms (Fig. 2). Each ommatidium receives light; the light is filtered through the lens situated on the outer surface of the eye. Further, the light passes the crystalline cone structure and then through the pigment cells and finally to the visual cells. Each ommatidium ends with its own nerve fiber which connects it to the common optic nerve. Each ommatidia relay its own information and form a tiny image. All the tiny images from each photoreceptor convalesce to form one visual image (Stavenga et al. 2005).

The camera eye of vertebrates produces an inverted image on the light-sensitive elements that is transmitted to the brain via optic nerves (Fig. 2). (Reviewed in Agi et al. 2014).

The phototransduction compartment, the light-guiding rhabdomere is formed by a stack of some 30,000 microvilli, each containing all the essential elements of the transduction cascade. Several elements of these cascades are common elements found in any phosphoinositol cascade, including the G-protein coupled receptor (rhodopsin), heterotrimeric G-protein (Gq), phospholipase C (PLCβ-4), and two closely related Ca^{2+} channels encoded by the *transient receptor potential* (*trp*) and *trp-like* (*trpl*) genes.

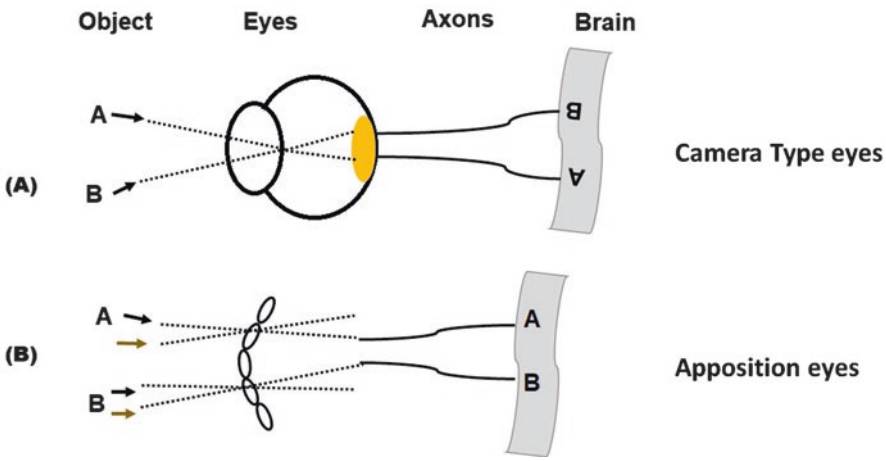

Fig. 2 Comparison of visual systems of vertebrate camera type eyes versus Drosophila apposition eyes. Light paths are shown as *dotted lines*. (**a**) The camera eye of vertebrates produces an inverted image on the light-sensitive elements that is transmitted to the brain via optic nerves. (**b**) Compound eye of *Drosophila* is an apposition type eye, which produces an upright image on the light-sensitive rhabdoms as well as in the first optic neuropil, the lamina. Image formed by individual ommatidium of the compound eye is an inverted image and only contributes a single pixel to the final image that is not further resolved. (Image adapted from Agi et al. 2014)

Development of Eye

The similarities and differences in compound eye of *Drosophila* versus camera type eye of vertebrates are due to the major differences and similarities of those hailed from embryonic or the developmental stages. Events at different developmental stages are tightly governed by the conserved genetic and molecular mechanisms which are common to both vertebrate and *Drosophila* eye development.

If it is only about developing an organ, such as an eye, both compound and camera type, what would be required? Assembly of cells, which will eventually differentiate into specialized structures of lens, retina, cornea, photoreceptors, rods, cones, pigment cells, accessory cells, and their neuronal connections to brain. Interestingly, for eye organogenesis, the classical processes of specification, determination, and differentiation follow the same processes for both flies and vertebrates.

Development of eye in both *Drosophila* and vertebrates begins at early embryonic stages. It is a fascinating process of converting a layer of cells into a three-dimensional functional organ involving axial patterning, followed by proliferation and differentiation. A pioneering research in the field of generation of axes during eye development has indicated that default *Drosophila* eye primordium is ventral, over which dorsal field is specified as the fly enters and proceeds to larval stages (Singh and Choi 2003; Singh et al. 2006, 2012, 2019). Once the dorsal-ventral axes are specified by specific axial patterning genes, cell proliferation is signaled.

Interestingly, these initial events are similar in the development of vertebrate eye as well, described below.

The early stages of vertebrate eye development have been revealed by several embryology experiments, which describe the morphological development of the early eye begins at embryonic day 8.5 (E 8.5), involving formation of an optic vesicle. The optic vesicle contacts head ectoderm to induce thickening of ectoderm forming lens placode. The lens placode invaginates and separates from surrounding ectoderm to form lens vesicle, while optic vesicle folds on itself inward, forming the optic cup. The lens vesicle cells eventually differentiate into lens structures, while optic cup cells form the neural and pigmented layers of the retina (Pei and Rhodin 1970; reviewed in Grainger 1992).

Drosophila eye primordium is ectodermal in origin, which is set aside as a group of only a few number of cells during embryonic stages. Studies have confirmed that the compound eye of *Drosophila* develops from population of embryonic primordial cells which converge to form anterior head segments, and develop into eye imaginal discs as early as first larval instar stage (Haynie and Bryant 1986; Jürgens et al. 1986; Green et al. 1993; Younossi-Hartenstein et al. 1993; Namba and Minden 1999; Chang et al. 2001; Huang et al. 2017). Imaginal discs are sac-like monolayer epithelial structures which form the blue prints for the adult organs in the *Drosophila*. The eye imaginal disc is a compound disc, which eventually differentiates into eye, antenna, and the head structures (Fig. 3) (Weismann 1864; Vogt and Anderson 1964; Gehring 1967; Ouweneel 1970; Baker et al. 1978; Haynie and Bryant 1986). During the first and second instar larval stages, eye disc cells divide almost homogeneously

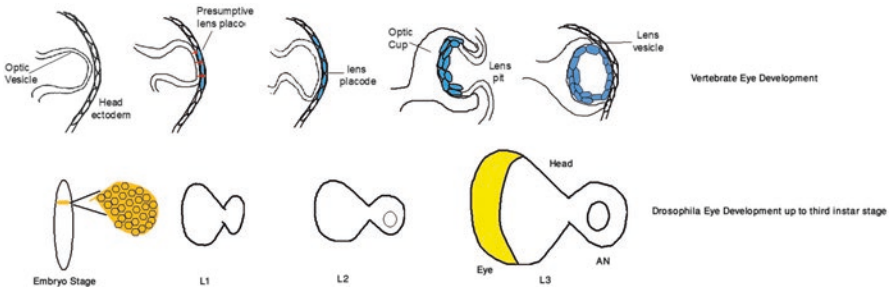

Fig. 3 Stages of eye development in vertebrates compared to *Drosophila*. (**a**) Eye development begins at embryonic day 8.5 in mouse. The optic vesicle forms a pouch like structure of the forebrain in the beginning, and contacts the head ectoderm on E9.0. Signals (indicated by red arrows), from optic vesicles induce formation of lens placode by E9.5. At E10.0, a few cells of lens placode (blue) invaginate to form a lens pit, whereas, optic vesicle forms an optic cup. The lens vesicle detaches itself from the ectoderm and invagination of lens pit gets completed by E10.5 to form the lens. Hereafter, the differentiation of the optic cup continues to form neural and pigmented epithelial layers of the retina. (**b**) Eye primordial cells are specified by ectodermal cells at an early embryonic stage. These cells proliferate in first and second instar larval stages (L1 and L2) to make a differentiated third instar (L3) eye antennal imaginal disc, which is a larval blue print for the adult eye, antenna and the head cuticle. The portion in yellow in L3 eye disc indicates the differentiated photoreceptor neurons which are separated from antenna and head through morphogenetic furrow (curved line)

and symmetrically by mitosis and imaginal disc grows bigger in size. However, at the end of second instar, or early third instar larval stage, mitotic divisions become asymmetric, for differentiation to begin. A stripe of *atonal* expression to recognize the R8 cells (*Math 5* in vertebrates) determines the apical constriction in posterior cells of the eye disc which appears like a furrow and moves towards the anterior of the eye disc. The stripe of *atonal* expression defining R8 cells, or the morphogenetic furrow (MF) rather moves like a Mexican wave in the football crowd (described by Jarman 2000). As the MF moves anterior, cells just ahead of it enter G1 arrest and stop proliferating. As cells are released from the furrow, they exit the cell cycle and begin differentiating as the R8, R2/R5/R3/R4 photoreceptor neurons of the precluster. A small subset will undergo a final round of mitosis (the second mitotic wave) before following their sister cells out of the cell cycle and into the ommatidium as the R1/R6/R7 photoreceptors, lens secreting cone cells, and optically insulating pigment cells (Ready et al. 1976; Wolff and Ready 1991; reviewed by Kumar 2018). A fully grown third instar eye disc (Fig. 3) contains antenna, head cuticle blue prints, in addition to differentiated photoreceptor neurons. This monolayer epithelial layer undergoes further changes into pupal stages, which include developing lenses, establishing neuronal connection with the brain, and acquiring pigments to appear a three-dimensional compound eye. After 36 h of pupariation, extra cells between the ommatidia are removed via apoptosis to form the regularly placed hexagonal facets.

It is intriguing that movement of MF in the *Drosophila* eye disc is required not only for differentiation, but also for regularly spaced photoreceptors; and is indeed similar to movements which occur in some of the vertebrates as well. The Mexican wave-like movement has also been demonstrated during eye development in zebrafish. Neurogenesis begins in optic cup epithelium, closer to optic stalk and then spreads outwards like a wave, which is controlled by *atonal* homolog *ath5*.

Genetic Regulation of Eye Development

The highly organized process of eye development is regulated by complex interplay of genetic networks. The advancements in the field of developmental genetics continue to demonstrate a high degree of genetic and molecular conservation during organogenesis of the eye, or oculogenesis between *Drosophila* and vertebrates. Many of the regulators of eye development were identified in *Drosophila* by gain-of-function and/or loss-of-function experiments before they were identified and characterized in vertebrate models. Molecular identities began to shine between two systems when *Pax6,* a member of Paired box family of transcription factor was found to be expressed initially in head ectoderm and optic vesicle, and then became restricted to lens placode ectoderm (Walther and Gruss 1991; Grindley et al. 1995). Despite the distinct morphological differences between the fly and vertebrate eyes, *Pax6* homologs, *eyeless* (*ey*) (Quiring et al. 1994) and *twin of eyeless* (*toy*) (Czerny et al. 1999) provide identity to the eye primordium. Out of two, *toy* is more similar

to Pax6 and acts upstream to *ey*. Both *Pax6* and *ey/toy* are capable of inducing ectopic eyes in most of the tissues upon overexpression and their mutations result in aniridia in mouse, and no eye phenotypes in flies (Ton et al. 1991; Glaser et al. 1992; Collinson et al. 2000; Quinn et al. 1996; Prosser and van Heyningen 1998; Quiring et al. 1994; Czerny et al. 1999; Halder et al. 1995). Several research labs have demonstrated that both *ey* and *toy* are expressed in other non-optic tissues as well, and therefore require other genes to induce the differentiation of the eye. Ectopic induction of ey can induce eye formation in the presence of *decapentaplegic* (*dpp*), a TGF-β family of growth factors (Heberlein et al. 1993; Chen et al. 1999). In addition to *ey* and *dpp*, other genes which are required for eye development are *Eyes absent* (*Eya*) (Bonini et al. 1993), *sine oculis (so)* (Cheyette et al. 1994), and *dachshund (dac)* (Mardon et al. 1994). Their vertebrate homologs are EYA 1/EYA2 (Zimmerman et al. 1997), Optix 2/Six 3 (Zuber et al. 1999), and Dach, respectively (Heanue et al. 1999; Ohto et al. 1999). These genes act in concert to aid in eye development (Fig. 4), and their mutations have been shown to cause defects in the eye development/visual impairment. Table 1 summarizes the comparative account on the genes involved in early events for eye development in *Drosophila* and vertebrates. It is noteworthy that genetic regulation is further accompanied by signaling events which are also conserved in vertebrates and *Drosophila*. For example, for differentiation of the eye primordium, downstream to *ey* additional signal from decapentaplegic pathway feeds in to initiate *eya* and *so*, which is actually a homolog of Bone Morphogenetic Protein-4/7 (BMP) in vertebrates. However, the difference between flies and vertebrates is, BMPs act in concert with *Pax-6* to induce lens placode, which eventually initiates the process of differentiation by inducing *Eya* and *Six-3*/*Optx-2* (reviewed by Chen et al. 1999).

Even though the initial events of the eye organogenesis are homologous in flies and vertebrates, the structural and anatomical differences (those discussed in previous sections) arise due to extremely complicated genetic networks, controlled by signaling events which are different in terms of spatiotemporal profiles, yet are governed similarly in the later stages of development which lead to formation of a three-dimensional eye.

Signaling aspect of cell–cell communication plays a major role in both vertebrate and *Drosophila* eye development. *Drosophila* equivalents of TGF-β, Sonic

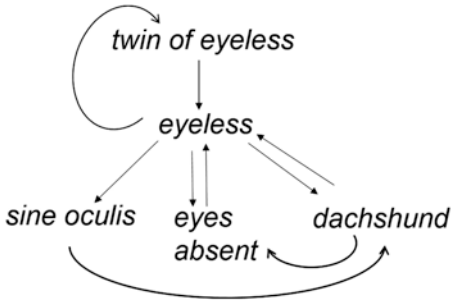

Fig. 4 Genetic regulation of eye development in *Drosophila*

Table 1 A comparative account of genes involved in retinal development in *Drosophila* and vertebrates (homologous domains of respective products have also been mentioned)

Genes	Vertebrates	*Drosophila melangoster*
Pax 6	Expressed in broad domain of head ectoderm and optical vesicle (Grindley et al. 1995).	*Pax 6* homolog ***ey (eyeless)*** restricted expression in cells anterior to morphogenetic furrow in a third instar imaginal disc (Quiring et al. 1994). *Pax 6* homolog ***toy (twin of eyeless)*** acts upstream of *ey* (Czerny et al. 1999). More orthologous to *Pax 6* due to a conserved C-terminal transcription activation domain.
Eyes Absent	***Eya1*** expressed in retinal pigment epithelium and optic nerve. Knockout of this gene cause severe optic abnormalities, cataracts (Azuma et al. 2000). ***Eya2*** expressed in neural retina, sclera and optic nerve sheath. ***Eya3*** expressed in the branchial arches and CNS, but lacks cranial placode expression (Xu et al. 1997). ***Eya4*** expressed primarily in the craniofacial mesenchyme, the dermamyotome and the limb (Borsani et al. 1999).	***Eya*** shares a highly conserved 271 amino acid regions at the C-terminus of the protein with the four homologs (Xu et al. 1997).
Dachshund	Human ***–DACH***. They have a homologous conserved domain called Dachbox –N and –C. -expressed in eye, limb, brain, neural tube, dorsal root ganglia, rib primordia and genital eminence (Hammond et al. 1998; Kozmik et al. 1999).	***Dac*** also contains the Dachbox which is homologous to the *Ski* and *Sno* family of oncogene-related proteins (Caubit et al. 1999).
Bmp	***Bmp4*** and the ***Bmp7*** gene co-express with *Pax6* in regulating eye formation and maintain lens placode development (Wawersik et al. 1999).	***dpp*** (***decapentaplegic***) a member of the TGF-β family of growth factors co-expresses with *ey* (Chen et al. 1998).
Six family	**Six 3**- member of six gene family are expressed in in-vaginating lens vesicle and developing retina. Mutations in this gene lead to microphthalmia and holoprosencephaly (Wallis et al. 1999).	***So*** (***Sine oculis***) have a conserved homeodomain and a stretch of 110 amino acids 5'to the homeodomain like six gene family (Ohto et al. 2002). *Eyeless* stimulates the expression of both *so* and *eya* gene (Halder et al. 1998).
Optx	*Optx2/six 6/six9:* Expressed only in optical vesicle and lens placode. It act as a fate determinant of retinal precursor cells that forms retinal neurons and photoreceptors (Toy et al. 1998). Humans- deletion of this gene lead to bilateral anophthalmia (Gallardo et al. 1999).	*Optix* gene- it is a true ortholog of *six3* gene and *optx2* gene. Expressed in early development of eye primordia and head (Toy et al. 1998).

Hedgehog, JNK, JAK STAT, EGFR, and Notch pathways have been widely studied in eye development as early as axes determination until sculpting the final organ shape (Greenwood and Struhl 1999; Roessler et al. 1996) (Fig. 4).

Concluding Remarks

Eye development is vast and has been studied widely to understand the processes of organogenesis and physiology by more researchers than we can think of. In the entire past century, the developmental biologists have elucidated basic framework of eye organogenesis in early and later stages, to understand the regulation and execution of these processes. With this framework aided with newer technologies such as 5D light sheet microscopy, newer forms of genetic manipulation techniques, and genome projects in *Drosophila* as well as vertebrate models, a converge understanding of regulators of eye development is being paved, which will aid the pre-existing knowledge to extrapolate the analogies between the two.

Acknowledgement Authors would like to thank Ms. Tripti Misra for arranging and commenting on the manuscript, in addition to Mr. Bhavsar to help with figure drawings. We apologize to the authors and publications that we could not include in our references.

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A. Singh, M. Kango-Singh (eds.), *Molecular Genetics of Axial Patterning, Growth and Disease in Drosophila Eye*,
https://doi.org/10.1007/978-3-030-42246-2