Understanding the mechanism of pigment rim formation at the periphery of the eye in *Drosophila melanogaster*

Sudha R. Kumar

Submitted in partial fulfillment of the

requirements for the degree of

Doctor of Philosophy

in the Graduate School of Arts and Sciences

COLUMBIA UNIVERSITY

2014

Abstract

Understanding the mechanism of pigment rim formation at the periphery of the eye in *Drosophila melanogaster*

Sudha R. Kumar

The *Drosophila* eye periphery undergoes peripheral patterning in response to a graded Wingless signal emanating from the surrounding head capsule. High levels of Wg signaling lead to the formation of the Pigment Rim. The pigment rim is a thick band of pigment cells that serves to optically insulate the eye from extraneous light rays. It is composed mainly of the pigment cells that surrounded the outermost row of ommatidia in the developing pupal eye. These peripheral ommatidia undergo timed developmental apoptosis, leaving the remaining pigment cells to coalesce and form the pigment rim. Earlier work showed that high levels of Wingless signaling induced the expression of Escargot, Wingless and Notum in a subset of the cells of the peripheral ommatidia, namely the cone cells. But the mechanism of apoptosis of the entire ommatidia remained unclear. My work focuses on the mechanism by which Wingless leads to the apoptosis of the different cell types of the ommatidia in a concerted manner.

In this thesis, I show that the peripheral apoptosis follows a precisely timed sequence of events. I also show that ectopic expression of Wingless at high levels causes the entire eye to respond in a manner similar to the peripheral ommatidia. In order to elucidate the mechanism of Wingless induced apoptosis, I analyzed the effects of manipulations of the Wingless signaling pathway in the subsets of the cells of the ommatidia. I found that the

expression of Escargot in the cone cells is required for their collapse, while the Wingless expression appears to be a booster signal for the apoptosis of the remaining cells of the ommatidia. I also show that the activation of Wingless signaling in the cone cells alone is insufficient for apoptosis of the ommatidia, thereby suggesting a combinatorial response of all the cell types. Lastly, I present a logical conundrum in the response of the photoreceptors to manipulations in Wingless signaling. In conclusion, I present a possible model of a concerted response of the different cell types of the ommatidia to lead to their apoptosis.

Table of Contents

List of figures for the Introduction chapter

List of figures for the Results chapter

Acknowledgements

First and foremost, I would like to thank my mentor, Andrew Tomlinson, for giving me the opportunity to pursue research in his lab. He has been a constant source of support and guidance through the years. He has taught me how to look beyond the obvious, and to examine data (mine, and others!) very critically before coming to conclusions. His intellectual criticisms and philosophical one-liners have gone a long way in helping me become a better scientist. And most importantly, I can't thank him enough for his patience with me, especially when the going got tough.

I'd like to acknowledge the members of the lab, current and former, who have helped me during these past years. This thesis would not have been possible without Yannis and Hina – who have been my support system from the day I joined the lab. They have been my friends, mentors, tormentors, critics, guides and family through the years. I would also like to thank Jason for safeguarding our stocks through dangerous times. I'd like to thank Gary Struhl for his help and generosity over the years. I'd also like to thank the members of the Struhl lab for their invaluable advice and encouragement. I thank all the people on the 11th floor of Hammer building. A walk down the hallway, or a few minutes in the conference room was all it took for gaining helpful advice as well as reagents.

I wish to thank the members of my thesis and qualifying committees for their time and advice: Dr. Richard Mann, Dr. Wes Grueber, Dr. Mimi Shirasu-Hiza, Dr. Iva Greenwald and Dr. Ginny Papaioannou. I'd also like to thank my rotation mentors Iva Greenwald and Laura Johnston for providing me with the opportunity to work with them. I thank the

administrative staff of the Genetics department: Nina Steinberg, Stacy Warren, Pascale Jean-Louis and Celia Marie Morales for all their help.

I'd like to thank my family, especially my mother, who has constantly supported me in every possible way. Also my brother, who introduced me to rock music, that helped me through many dissections. I consider myself extremely lucky to have found myself surrounded by friends and family in a new country. Thank you all for helping me retain my sanity and optimism. Last but not the least; I would like to thank my husband for his unwavering support and love. Without you all, this would not have been possible.

Introduction

Pattern formation by morphogen gradients

The development of multicellular organisms is a complex process, involving cell proliferation, differential gene expression and regulation, inter-cellular interactions and growth. For example, the Drosophila wing disc develops into a stereotypical pattern of wing veins, bristles, and hairs. On the other hand, the limb primordia for human arm develop a hand with five distinct digits placed in a stereospecific pattern. Trying to understand process of spatial patterning of an initially homogenous tissue to form diverse structures has been a key research area for developmental biology. One of the initial insights into patterning of tissues came from the seminal work done by Hans Spemann and Hilde Mangold in 1923. In their experiment, they transplanted dorsal lip tissue from an early newt gastrula into another early gastrula in a region originally fated to become the ventral epidermis. However, the transplantation led to mirror-image duplication of the whole body (translated in (Spemann and Mangold 2001)). This led to the key observation that a small cellular cluster, called the 'organizer' regions, possessed the ability to induce developmental fates in the surrounding tissues. It also led to the hypothesis that there must be an 'instructive' signal emanating from such organizer centers that led to pattern formation. These observations spurred a series of embryological experiments in a variety of model organisms, in order to try and discover the 'organizing center' and the signaling mechanisms which lead to the patterning of tissues.

1.1 Morphogens

The postulated signaling molecules released from the organizing centers were termed 'morphogens' – meaning form generating substances (Turing, 1952). Ideally, a morphogen should be secreted from a localized 'source', and form a gradient as it moves away from the same. This gradient should be able to induce distinct cellular responses in a threshold dependent manner, which would lead to the formation of discrete tissue subtypes. Any changes in the concentration of the morphogen should be paralleled by the concordant changes in cellular responses. And finally, the morphogen should be able to elicit all the graded responses directly, not via intermediary signaling molecules. Based on these criteria, some of the most extensively studied candidates for morphogen activity include the signaling proteins of the Wnt, Hedgehog(Hh), Fibroblast Growth Factor(FGF), Bone Morphogenetic Protein(BMP), Epidermal Growth Factor(EGF) and Transforming Growth Factor-β(TGF-β) families(reviewed in(Gurdon and Bourillot 2001).

1.2 Establishing morphogen gradients

Although a lot of work has been focused on the identification and characterization of morphogens, there is no consensus yet on the mechanism by which the morphogen gradients are formed and maintained. The simplest hypothesis proposed for a linear gradient formation was free diffusion of the morphogen across a few cell diameters through the extracellular matrix (Crick 1970). However, this explanation does not address the facts that most of the known morphogens are hydrophobic, and they often act over long ranges. Additionally, morphogen diffusion might be influenced by the presence of

morphogen-binding receptors or by post-translational modifications by heparin sulfate proteoglycans (HSPG) (Lander, Nie et al. 2007, Yu, Burkhardt et al. 2009, Lei and Song 2010). Taking these factors into account, recent experimental and theoretical studies use a restricted diffusion or hindered diffusion model to explain the formation of morphogen gradients (reviewed in (Muller, Rogers et al. 2013, Yin, Wen et al. 2013).

Alternative mechanisms to explain stable, long-range gradient formation involve the cellularization and transport of the morphogens. Transcytosis involves repeated cycles of endocytosis, secretion and intra-cellular trafficking of the signaling molecule to move it away from the source. Long-range gradient formation of Decapentaplegic (Dpp) has been known to require transcytosis (Entchev, Schwabedissen et al. 2000, Kicheva, Pantazis et al. 2007). Argosomes are exosome-like vesicles involved in the packaging and dispersal of signaling molecules. The establishment of Wingless (Wg) gradient along the dorso-ventral axis of the developing fly wing involves argosome formation (Greco, Hannus et al. 2001). Cytonemes are actin-based filopodial projections sent out by the cells in the direction of the source to directly receive the morphogen signal (Ramirez-Weber and Kornberg 1999). Cytoneme-based gradient establishment is involved in the Dpp gradient formation in the wing, Spitz signaling in the developing eye disc and FGF signaling in the trachea (Hsiung, Ramirez-Weber et al. 2005, Roy, Hsiung et al. 2011) reviewed in (Gradilla and Guerrero 2013). Although more experimental evidence is needed, the current research suggests that a combination of these strategies is utilized to set up the various morphogen gradients.

1.3 Morphogen gradient interpretation

Cells in a tissue receive positional information from the morphogen in order to initiate the appropriate developmental programs. The interpretation of the information conveyed by a morphogen gradient can be explained using Wolpert's French flag model (Wolpert 1971). In this model, a line of cells subjected to a graded signal can differentiate into Red, White or Blue cells depending on their position within the gradient (Fig.1).

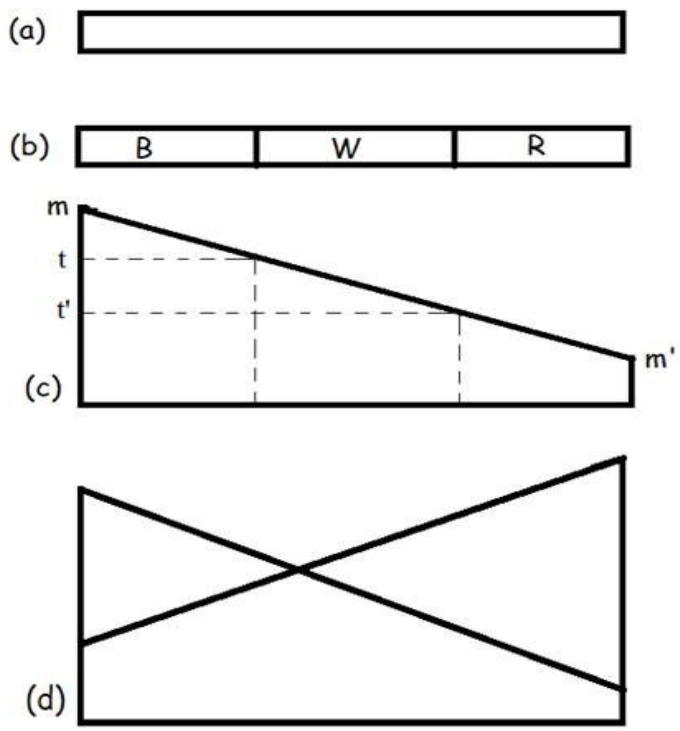

Figure1. Wolpert's French Flag model

A line of cells (a) undergoes differentiation such that a third of the cells each form the

Blue (B), White (W) and Red (R) compartment, as seen in (b). This can be accomplished by a linear gradient (c) with fixed boundary values m, m' and variable threshold values t, t'. Alternatively it can be due to two opposing gradients (d), the thresholds determined by the relative ratio of the gradients. Figure adapted from (Wolpert 1971).

In this case, the positional information is assigned to the cells via a linear gradient with fixed boundary concentration values m, m' and rules of interpretation are based on the threshold values t and t' (Figure 1(c)). An alternate way of assigning the positional information is to have two opposing gradients, with the ratio of the concentration of the two signaling molecules being the differentiation determinant (Figure 1(d). Depending on their position in the gradient, the cells interpret the information and activate the appropriate genes required to form the Red, White or Blue cell type. The critical point here is that the positional value conferred on a cell is the determinant of its final differentiated state (Wolpert 1971). The information derived by the cells consists of two components: the scalar component and the vector component. The scalar component provides information about the absolute concentration of the morphogen, while the vector component provides directional input to allow cell orientation. In this thesis, we shall be dealing only with the scalar input derived from the gradient.

1.4 Examples of morphogen gradients in development

A variety of morphogen gradients have been described in different model organisms. Sonic Hedgehog (Shh) has been implicated in the patterning of the vertebrate neural tube. It has been shown that different neuronal populations arise at distinct locations along the

dorso-ventral axis of the neural tube, in response to graded Shh signaling (Ashe and Briscoe 2006, Cohen, Briscoe et al. 2013). Nodal, a member of the BMP family of proteins has been implicated in mesendoderm patterning in frogs and zebrafish (Feldman, Gates et al. 1998, Dougan, Warga et al. 2003). Nodal transcription is restricted to the vegetal region of the embryo, which overlaps the endoderm precursors and is adjacent to the mesoderm precursors. This spatially restricted gradient of Nodal signaling has been shown to induce endoderm patterning at high levels, and mesoderm patterning at lower levels away from the source (reviewed in (Schier 2009)).

In addition to the vertebrate models, *Drosophila melanogaster* embryos have been studied extensively for patterning in response to morphogens. The first proteins identified as morphogens, were Bicoid and Hunchback, which are transcription factors acting early in the syncytial embryo to lead to patterning. A more general field for studying patterning in response to morphogen gradients are the developing imaginal discs. In the developing wing disc, Dpp was shown to be the morphogen responsible for patterning along the anterior-posterior axis of the wing (Nellen, Burke et al. 1996).

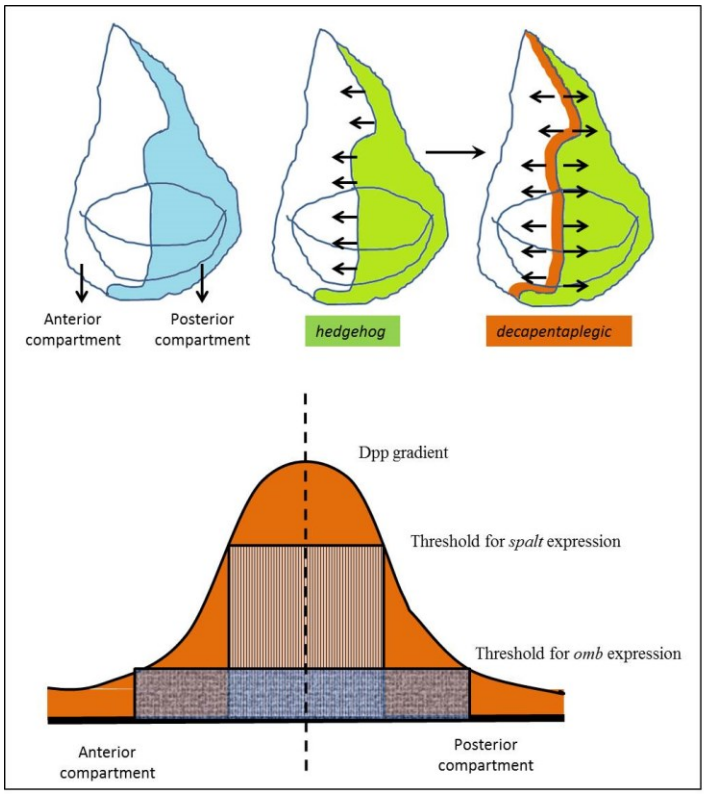

Figure 2: Dpp morphogen gradient patterns the developing wing imaginal disc along the A/P axis Hh is expressed in the posterior compartment of the developing wing disc. This induces the expression of the morphogen Dpp in a stripe of cells immediately anterior to the A/P compartment boundary. The graded Dpp signaling leads to threshold dependent induction of target genes *spalt* and *optomotorblind (omb).*

In response to Hh signaling in the posterior compartment, Dpp expression is activated in the anterior cells adjacent to the compartment boundary. This leads to the expression of *spalt* in a region flanking the Dpp source, and a broader domain of *optomotor-blind* expression in a threshold dependent manner (Nellen, Burke et al. 1996). Similarly Wg

has been proposed to act as the morphogen for dorso-ventral patterning of the wing disc (Zecca, Basler et al. 1996).

We will also be utilizing Drosophila as the model organism in order to understand patterning by Wg at the periphery of the retina during pupal development.

1.5 Life cycle of *D. melanogaster* (The fruit fly)

D. melanogaster is a holometabolous insect of the order *Diptera* i.e. the life cycle goes through four stages, embryo, larva, pupa and the adult. We shall be utilizing *D. melanogaster* as the model organism for studying peripheral patterning in the retina. The key benefits of using the fruit fly are its ease of maintenance, wide variety of scientific tools available and a relatively shorter life cycle.

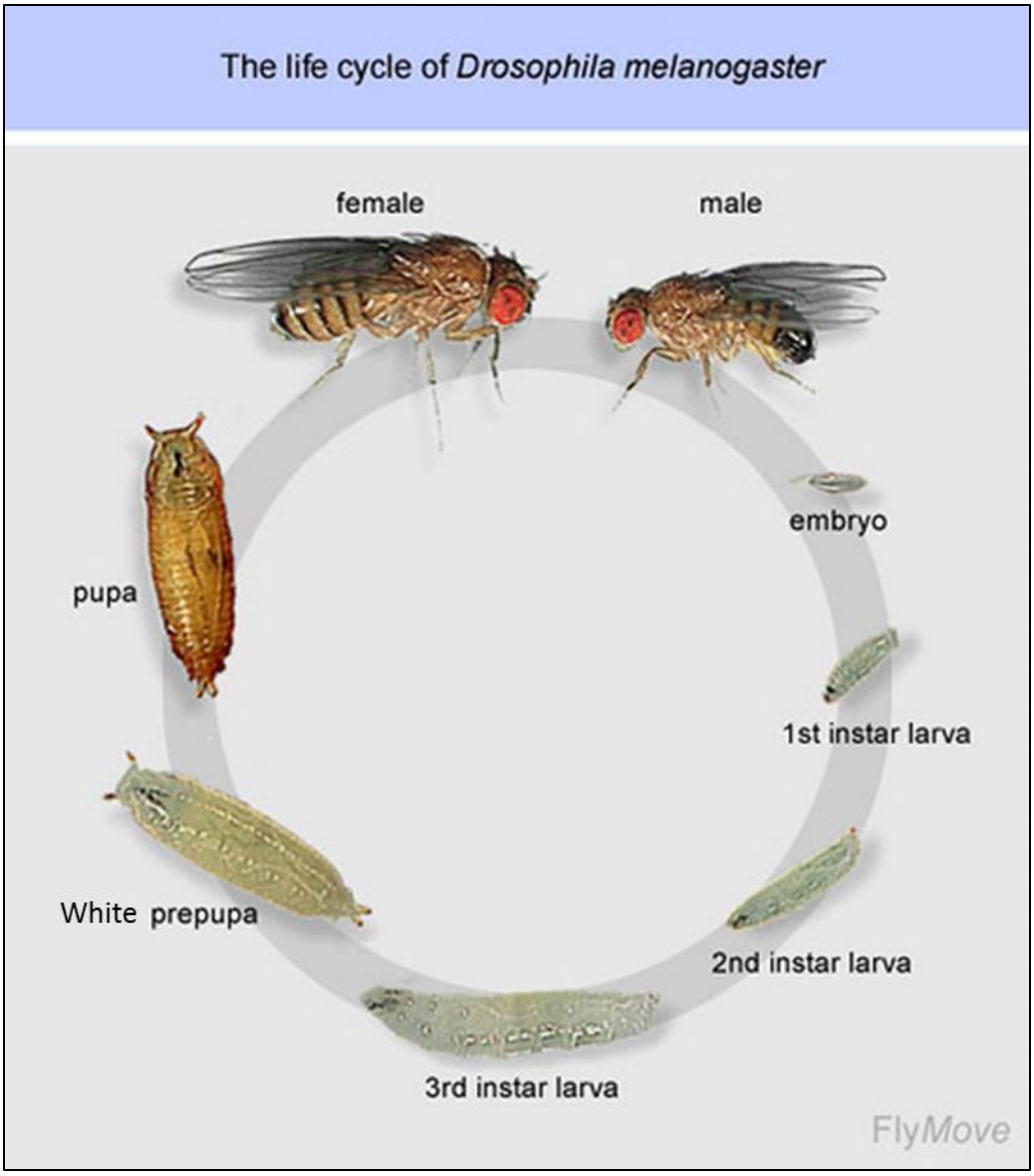

Figure 3: The life cycle of *Drosophila melanogaster*

The life cycle goes through four stages: embryo, larvae, pupae and adult. The individual stages are described in detail in the text. Image taken from (Weigmann, Klapper et al. 2003)

The life cycle takes about 10 days to complete at 25°C room temperature. Once fertilized, the embryo hatches in 24 hours and then undergoes successive molts, referred to as the larval instars. The precursors for the adult organs are set aside during early embryonic stages as groups of imaginal cells. These cell clusters form inverted epithelial sacs called imaginal discs that undergo growth and patterning during the larval stages, and evert during the pupal stage to form the adult structures. The larval stages are thus characterized by feeding and growth. At the end of the third larval instar, the larvae undergo pupariation.

The larval growth stages are referred to as the number of hours After Egg Laying (AEL). The onset of the pupal stage is marked by the formation of the white pre pupae (wpp). Pupal developmental time points are referred to as the number of hours After Puparium Formation (APF). The pupal stage is quiescent, and involves the degeneration of the larval structures and eversion and maturation of the imaginal discs. The adult fly ecloses after about 5 days of pupal development (Weigmann, Klapper et al. 2003, Ashburner and Roote 2007).

The most compelling advantage of using Drosophila as the model organism derives from the immense body of research material accumulated over more than a century, freely available to the members of the research community. Drosophila has only 3 pairs of autosomes and one pair of sex chromosomes. Meiotic recombination is suppressed in males, and the availability of balancer chromosomes (which carry multiple inversions, thus preventing recombination) allows the maintenance of stocks carrying lethal

mutations. With the onset of molecular biology, a wide variety of genetic and molecular tools became available to researchers to enable them to conduct unbiased genetic screens as well as to manipulate gene expressions in the desired manner (Roberts 2006, Neckameyer and Argue 2013). A major advantage of using Drosophila to identify components of various signaling pathways is that expression of lethal components can be targeted to non-vital organs such as the eye and the effect of subsequent manipulations can be analyzed.

There are a wide variety of scientific tools available, including a range of aneuploidy strains (carrying a deficiency or duplication in a chromosomal locus), classical mutations and various transgenic lines. A key tool is the bipartite UAS-Gal4 system (Brand and Perrimon 1993) which allows tissue specific expression of target genes. With the generation of UAS-RNAi lines, which encode hairpin RNAi to various genes, it is possible to cause tissue specific knockdown of genes at various developmental time points. The temporal expression can also be controlled by adding a 'heat-shock' promoter to our driver lines, thus enabling stage specific ectopic expression (Clos, Westwood et al. 1990). Another binary system, similar to the UAS-Gal4 system is the LexA/LexO binary trans-activator system (Yagi, Mayer et al. 2010). Together these tools provide us the ability to finely manipulate gene expression within the normal expression areas, as well as in a foreign milieu.

2 Wingless

The *wingless* gene was first identified by Sharma, R.P. *wg* mutant flies lack wings (hence the name) and halteres, instead these flies show a duplication of notum structures (Sharma and Chopra 1976). *wg* was initially classified a segment polarity gene, as zygotic mutations interfered with embryonic patterning (Nusslein-Volhard and Wieschaus 1980). Later on, Wg signaling was shown to be involved in many developmental and patterning processes.

The Int-1 gene was identified as a proto-oncogene for mammary tumors in mice (Nusse and Varmus 1982). Wnt genes (jointly referring to Drosophila Wg and mammalian Int-1 genes) are a diverse family of evolutionarily conserved genes coding for lipid-modified, secreted glycoproteins. These proteins have a signal sequence, followed by a highly conserved distribution of cysteine residues which undergo various post-translational modifications. Unlike most secreted factors, Wnts are not freely soluble in the extracellular matrix. The various lipid modifications enable membrane tethering and influence the degree of diffusibility of the Wnt proteins (Willert, Brown et al. 2003).

Besides *wingless,* there are 6 other D-Wnt genes (D-Wnt 2, 3/5, 4, 6, 8, 10) identified in Drosophila. They have been reported to be involved in many developmental processes, for instance, DWnt2 in the development of trachea and the male reproductive tract (Kozopas, Samos et al. 1998, Llimargas and Lawrence 2001), and DWnt3/5 in axon guidance in embryonic CNS development (Fradkin, Noordermeer et al. 1995, Fradkin,

van Schie et al. 2004). But *wingless* is the most extensively studied DWnt gene in *Drosophila*.

2.1 Wingless signaling pathway overview

There are two pathways of Wg signaling reported in *Drosophila* – the Wnt/β-catenin pathway, also referred to as the canonical pathway(reviewed in (Logan and Nusse 2004)); and the planar cell polarity(PCP) pathway, also called the non-canonical pathway(Veeman, Axelrod et al. 2003). The Frizzled (Fz) receptor and Dsh interaction at the plasma membrane is common to these pathways (Rousset, Mack et al. 2001). A third pathway involving calcium levels and calmodulin-dependent kinases, leading to Wnt-dependent cell adhesion changes has been reported in vertebrates, but does not exist in Drosophila(Kuhl, Sheldahl et al. 2000, Kuhl, Sheldahl et al. 2000).

The non-canonical (PCP) pathway has been extensively utilized to direct cellular orientation in tissue patterning- for e.g. wing hair orientation (Mitchell, Stubbs et al. 2009) and ommatidial rotation in the eye (Das, Reynolds-Kenneally et al. 2002). Downstream activation of this pathway utilizes a new set of cytoskeletal reorganization proteins including VanGogh (Vang), Flamingo (Fmi), Starry night (Stan) and Prickled (Pk). These proteins are recruited to the plasma membrane to form a multi-protein

complex which ultimately leads to cytoskeletal remodeling (reviewed in (Veeman, Axelrod et al. 2003)).

We shall be dealing only with the canonical Wg signaling pathway in this thesis, with special emphasis on the genes we examined for our studies – Arm, Axn and APC.

2.2 Wnt/β-catenin canonical signaling pathway

The key step of the canonical Wg signaling pathway is the stabilization of Armadillo (Arm) - the fly homolog of β-catenin. In the absence of Wg ligand, Arm is targeted by the degradation complex – consisting of Adenomatous Polyposis Coli (APC), Axin (Axn), Casein Kinase 1α (CK1α) and Shaggy (Sgg) – the fly homolog of Glycogen Synthase Kinase-3 (GSK-3) (Seto and Bellen 2004). APC and Axn act as scaffolding proteins while CK1α and Sgg phosphorylate Arm at the N-terminus, thus promoting its ubiquitination and degradation (Hart, de los Santos et al. 1998, Ikeda, Kishida et al. 1998). Wg signaling is mediated by its receptors Fz / DFz2 (Bhanot, Fish et al. 1999) and the co-receptor Arrow (LRP5/6). The binding of Wg to its receptors causes recruitment of the cytoplasmic protein Dishevelled (Dsh) to the plasma membrane and the phosphorylation of Arrow at PPPS/TP motifs by Sgg (Tamai, Zeng et al. 2004, Zeng, Tamai et al. 2005). This phosphorylation promotes docking of Axn to the plasma membrane, thus disrupting the degradation complex (Mao, Wang et al. 2001, Tolwinski, Wehrli et al. 2003). This allows the stabilization and accumulation of Arm in the cytoplasm and in the nucleus. In the nucleus, Arm can now interact with Pangolin (Pan) – the fly homolog of TCF/LEF family of transcription factors. Pan binds DNA via its High

Mobility Group (HMG) domain. In the absence of Wg, Pan interacts with Groucho and CBP (cyclic AMP response element binding protein) to cause repression of Wg target genes (Cavallo, Cox et al. 1998, Waltzer and Bienz 1998). In the presence of Wg, the Arm/Pan complex acts as a co-activator for the transcription of Wg target genes (DasGupta, Kaykas et al. 2005).

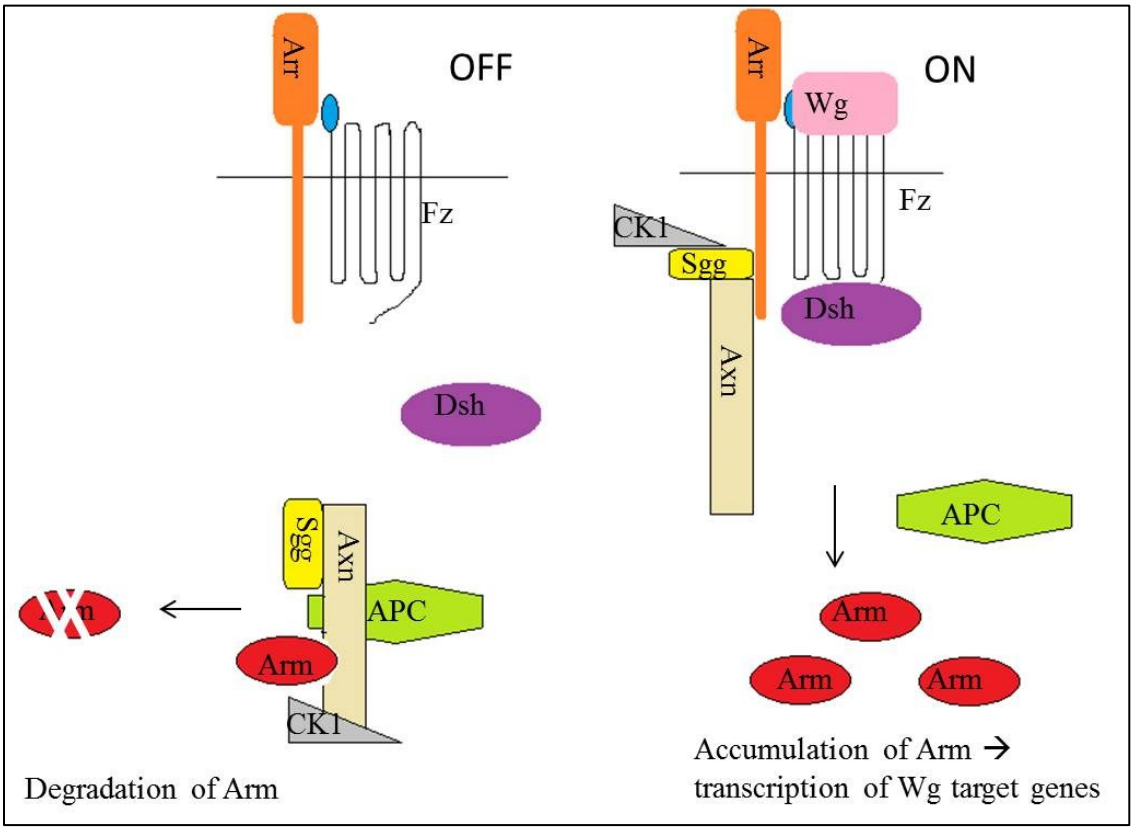

Figure 4: Canonical Wingless signaling pathway

In the absence of Wg stimulation, steady-state levels of Armadillo (Arm) are maintained via its constitutive synthesis and proteolysis. The Axin (Axn) scaffold facilitates the

association of Arm, Shaggy (Sgg), CK1α, and APC. Phosphorylation of Arm by CK1α and Sgg promotes its

phosphorylation and proteasomal degradation. In the presence of Wg, there is formation of a

receptor complex between Frizzled (Fz), Arrow (Arr) and Wg, leading to recruitment of Dishevelled (Dvl) to Fz. Formation of this complex triggers phosphorylation of Arr by Sgg and CK1α, and the subsequent recruitment of Axin and Sgg to the Fz-Arr receptor complex. Formation of this signaling complex results in inactivation of the destruction complex, leading to Arm stabilization, nuclear translocation, and Wg target gene activation. Figure adapted from (Tacchelly-Benites, Wang et al. 2013)

2.3 Members of the canonical Wg signaling cascade

2.3.1 Wg receptor complex – Fz/Arrow/Dsh

Upon receiving the Wg signal, two distinct cell surface receptors respond together to initiate signaling. The first is the Frizzled class of serpentine receptors, and the second are the Arrow transmembrane proteins. Frizzled (Fz) receptors constitute the family of G-protein coupled seven-pass transmembrane proteins, with a conserved Cysteine Rich Domain (CRD) and a conserved S/T-X-V sequence at the C-terminus (Bhanot, Brink et al. 1996). There are 4 Fz receptors known in *Drosophila:* Fz, DFz2, DFz3 and DFz4. DFz was identified as a Wg receptor in mediating planar cell polarity (Vinson and Adler 1987, Bhanot, Fish et al. 1999). DFz2 is a Wg receptor with a much higher affinity for Wg, and

is transcriptionally up regulated in response to Wg signaling (Bhanot, Brink et al. 1996, Tomlinson, Strapps et al. 1997). DFz3 and DFz4 have not been directly implicated in Wg signaling response (Sato, Kojima et al. 1999, Rhee, Sen et al. 2002).

Binding of Wg to the Fz receptor leads to the clustering of the co-receptor Arrow (Arr). Arr is a single transmembrane spanning protein of the LDL-related receptor protein (LRP) family (Tamai, Semenov et al. 2000, Wehrli, Dougan et al. 2000). The phosphorylation of Arr at its key PPPS/P residues is critical for its activation (He, Semenov et al. 2004, Tamai, Zeng et al. 2004). Experiments performed using chimeric Fz-Arr fusion receptors and artificially dimerised Arr receptors suggest that Wg signal activation is a two-step process – Initiation, which requires Fz/Arr co-activation, and amplification which is dependent on clustering of Arr proteins at the membrane (Baig-Lewis, Peterson-Nedry et al. 2007). The initiation step is postulated to be involved in the recruitment of Dsh to the membrane. This Dsh recruitment has been shown to be crucial in planar cell polarity (Wong, Bourdelas et al. 2003, Wu, Jenny et al. 2008). In addition, this Fz-Dsh interaction might also be involved in recruiting Axn and Sgg to the membrane, thus promoting pathway activation via Arr phosphorylation (Kishida, Yamamoto et al. 1999, Cliffe, Hamada et al. 2003).

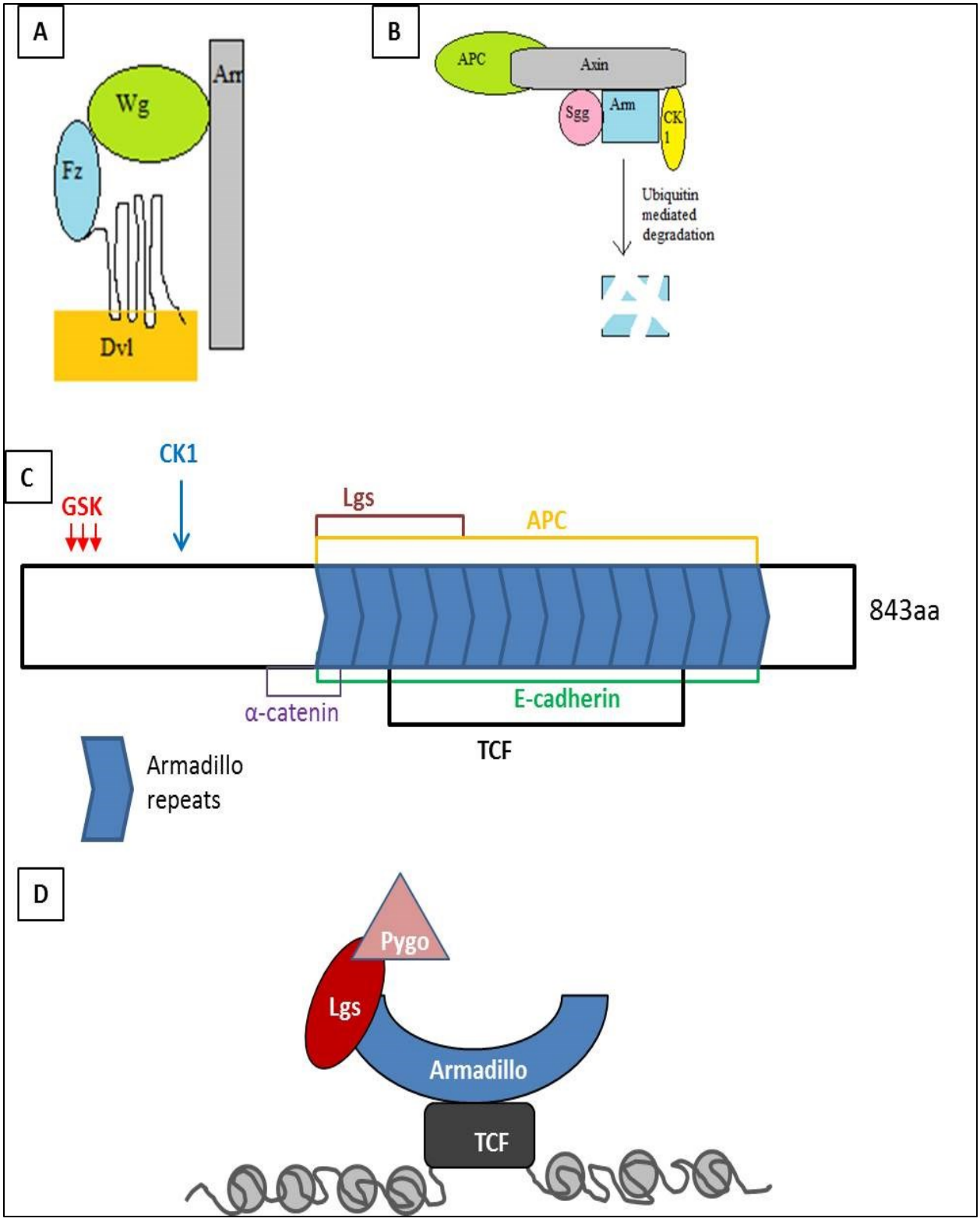
A
Wg
Arr
Fz
Dvl
B
APC
Axin
Sgg
Arm
CK
1
Ubiquitin
mediated
degradation
C
CK1
GSK
Lgs
APC
843aa
α-catenin
E-cadherin
TCF
Armadillo
repeats
D
Pygo
Lgs
Armadillo
TCF

Figure 5: Structures of the members of the Arm Degradation complex

A: The Fz-Arr-Dsh receptor complex : Frizzled (Fz) receptors are composed of a cysteine rich domain (CRD) at the N terminus to which Wg binds, a hydrophilic domain region of 40-100 amino acids, 7 transmembrane domains and conserved sequences at the C terminus. Dishevelled

(Dsh) binds to Fz in the first and third intracellular loops. Arrow (Arr) is a single pass transmembrane receptor (adapted from MacDonald et al, 2009).

B: In the absence of Wg, Armadillo is phosphorylated and ubiquitinated in the degradation complex for subsequent proteasomal degradation. Axin is the scaffold for the degradation and binds to APC, Armadillo, Sgg and CK1. APC in conjunction with Axin mediates the assembly of the degradation complex and capture of Armadillo. Sgg and CK1 phosphorylate Armadillo priming it for ubiquitination.Wingless signaling disassembles the degradation complex thus stabilizing Armadillo. (Adapted from Huang & He, 2008).

C: At the N terminus of Armadillo/β-catenin are the sites of Sgg/CKI phosphorylation, needed for its degradation. The central region consists of twelve armadillo repeats that bind proteins involved in Wnt signaling and cell adhesion. E-cadherin, involved in cell adhesion, binds to all twelve Armadillo repeats. In the degradation complex APC also occupies the twelve armadillo repeats. For signaling, TCF binds to eight central Armadillo repeats, while Lgs binds to the first four repeats (Adapted from Bienz, 2005).

D: In the nucleus, transcription activator Pygo is recruited to TCF bound Armadillo/β-catenin by

Lgs/BCL9 and binds N terminally.

2.3.2 The Degradation complex – This complex is responsible for maintaining low levels of free cytosolic Arm. The excess Arm is targeted for degradation by the concerted action of Axn, APC and Sgg.

Armadillo – the fly β-catenin

armadillo (a*rm*) gene was identified as a segment polarity gene in a screen for genes involved in proper patterning of the posterior segments of the *Drosophila* embryo (Nusslein-Volhard and Wieschaus 1980). Mutations in the *arm* gene caused nearly identical embryonic phenotypes as that of *wg* mutant embryos. Further work demonstrated that Wg regulates Arm accumulation in a post transcriptional manner (Riggleman, Wieschaus et al. 1989, Riggleman, Schedl et al. 1990). Arm was also reported to be the *Drosophila* homolog of the β-catenin family of proteins (Peifer and Wieschaus 1990). These β-catenin proteins were identified as members of mammalian cadherin complexes, involved in the adhesion of cytoplasmic actin filaments to the cadherin complexes (McCrea, Turck et al. 1991). Thus these proteins were among the first examples of proteins being involved in multiple distinct cellular processes.

Arm is a highly conserved, functionally modular protein, made up of 843 amino acids. The protein structure can be divided into three domains: an acidic N – terminus of about 139 amino acids, a central 'ARM domain' of 576 amino acids containing 13 imperfect repeats of about 42 amino acids each (referred to as 'arm repeats'), and a C- terminus made of 128 amino acids which is rich in Pro and Gly residues (Riggleman, Wieschaus et al. 1989, Peifer and Wieschaus 1990). The tandem repeats in the central ARM domain contain three helices each, and together they form a rigid rod-like super-helix of helices (Huber, Nelson et al. 1997). This rigid domain has a slight curvature, which enables interactions with various binding partners on the inner concave side of this domain (Gottardi and Peifer 2008). The N-terminal region is responsible for stabilization of the Arm protein. It contains a series of Ser/Thr residues, which can be phosphorylated, thus targeting the protein for proteasomal degradation. Ectopic expression of mutant versions of the Arm protein lacking the phosphorylation sites leads to chronic, constitutive activation of Wg signaling in the cells. The C-terminal domain of Arm acts as a strong trans-activator domain upon binding to DNA. It has been reported that the C-terminal domain of β- catenin alone, upon being fused to (TCF/LEF) transcription factors, is sufficient to cause transcription of Wnt target genes (Vleminckx, Kemler et al. 1999).

In the absence of Wg signaling, Arm levels in the cytoplasm are tightly regulated by targeting the excess molecules for degradation. Arm is phosphorylated at Ser-45 by the priming kinase CK1α; this allows Sgg to phosphorylate Arm at Ser-33, Ser-37 and Thr-41 (Kimelman and Xu 2006). This phosphorylated N-terminus of Arm acts as a substrate for the E3 ubiquitin ligase (β-TRCP1), thus targeting Arm to the proteasome for

degradation (Kikuchi and Kishida 2006). Upon Wg ligand binding, activation of target gene transcription by Arm in the nucleus is mediated via interaction with Pangolin (Pan), and CBP (Takemaru and Moon 2000). The complete transcription co-activator complex requires additional proteins like Legless (Lgs) and Pygopus (Pygo) (Townsley, Cliffe et al. 2004).

In addition to its role as the key transducer of Wg signaling, Arm also plays a role at the cell junctions by interacting with DE-cadherin and α-catenin. Overlapping regions of Arm are required for its role in signaling as well as adhesion functions. Hence the competition for binding by its partners may direct the relative levels of Arm in the nucleus or at the plasma membrane (Cox, Kirkpatrick et al. 1996, Orsulic and Peifer 1996, Brembeck, Schwarz-Romond et al. 2004).

Axin – the scaffolding protein

Axin (Axn) serves as the scaffold for the assembly of the Arm degradation complex.

Mutations in Axn have been reported to lead to stabilization and accumulation of Arm and downstream Wg target genes (Hamada, Tomoyasu et al. 1999, Kawahara, Morishita et al. 2000). Ectopic expression of Axn has been reported to reduce nuclear Arm by binding and sequestering it at the membranes, thus reducing Wg signaling in the cell (Mendoza-Topaz, Mieszczanek et al. 2011). Full length Axn has been reported to enhance the phosphorylation of Arm by binding a groove in Sgg (Ikeda, Kishida et al. 1998, Dajani, Fraser et al. 2003).

D-Axn was initially discovered in a yeast two-hybrid screen for Arm-binding proteins (Hamada, Tomoyasu et al. 1999). Structural analysis revealed that D-Axin has significant homology to the mammalian Axin family proteins, that were reported to be involved in negative regulation of the Wnt signaling pathway (Zeng, Fagotto et al. 1997).

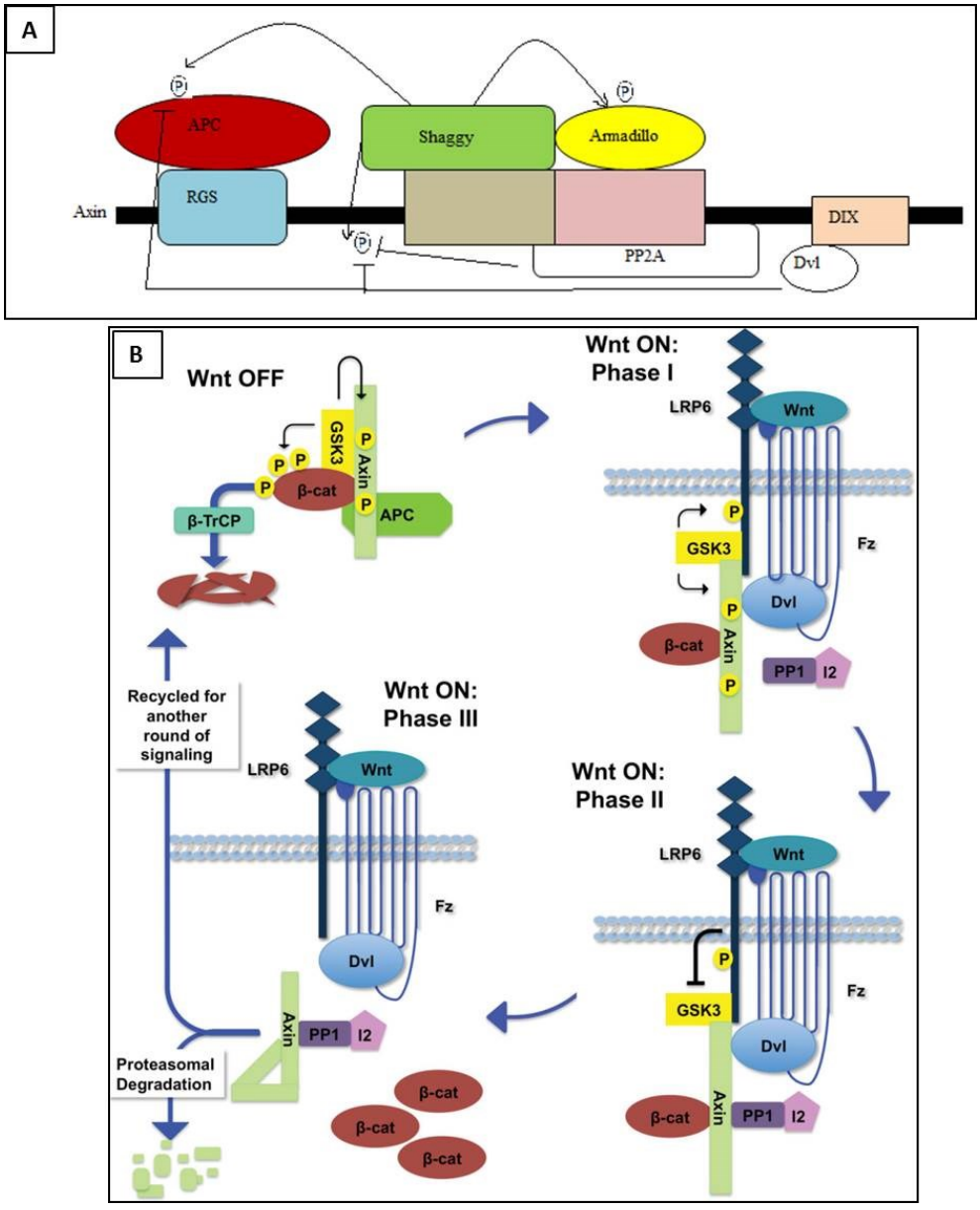

Figure 6: Axin – the scaffolding protein

A: Axin is the scaffold of the degradation complex. APC binds to Axin via a conserved RGS domain. Their interaction mediates the capture of Armadillo/β-catenin and formation of a ternary

complex. Sgg and CK1 phosphorylate Axin, APC and Armadillo/β-catenin. Phosphorylation of APC regulates its affinity for Armadillo/β-catenin while phosphorylation of the latter primes it for degradation. At the C terminus Axin has a conserved DIX domain, which permits Axin polymerization required for its function and dimerization with Dsh, which destabilizes the degradation complex. Dephosphorylation of Axin by PP1 also destabilizes the complex by impairing Axin's interaction with Sgg (Adapted from Kikuchi, 1999).

B: The axin phosphorylation / dephosphorylation model proposed to explain role of Axin as the sensor for Armadillo/β-catenin levels in the cell. In the absence of Wnt signaling, the open conformation of Axin promotes the formation of the degradation complex. In response to Wnt signaling, Axin gets dephosphorylated, thus promoting the closed conformation and its degradation by the proteasome machinery. Figure taken from (Tacchelly-Benites, Wang et al. 2013)

It was also shown that Axn has distinct binding domains for binding to different members of the Wg signaling pathway –

a) a conserved N-terminal RGS (regulator of G-protein signaling) domain that interacts with D-APC; b) a central β-catenin binding domain (BCD); and c) a C-terminal DIX domain reported to be required for Axn polymerization, which is essential for the protein to function as a part of the degradation complex (Nakamura, Hamada et al. 1998, Hamada, Tomoyasu et al. 1999).

The C-terminal DIX domain is also able to dimerise with a similar DIX domain in Dsh. This interaction is believed to recruit Axn to the plasma membrane in the presence of Wg (Fiedler, Mendoza-Topaz et al. 2011).

Axn is present in the cells at much lower concentrations than the other members of the degradation complex, thus the levels of Axn act as the limiting factor for the amount of Arm targeted for proteolytic degradation (Behrens, Jerchow et al. 1998, Ikeda, Kishida et al. 1998, Lee, Salic et al. 2003). Axn is also phosphorylated by Sgg at Ser-497 and Ser-500; these phosphorylation events increase the activity of Axn in the degradation complex, along with promoting its stability (Jho, Lomvardas et al. 1999, Yamamoto, Kishida et al. 1999).

Upon Wg stimulation, Axin is dephosphorylated by PP1γ, a ubiquitous Ser/Thr phosphatase. This dephosphorylation is believed to reduce the stability of axin and its affinity for Arm (Luo, Peterson et al. 2007). This phosphorylation-dephosphorylation of Axn is closely associated with the accumulation of Arm levels in the cell following Wg stimulation.

As Axn is a key scaffold protein involved in both the degradation complex as well as the signaling complex, He et. al. decided to examine the role of Axn phosphorylation in the switching of its role between the On/Off states of the pathway (Kim, Huang et al. 2013). Based on their studies of association of β-catenin with Axn before and after Wnt stimulation, He and colleagues propose the following model for the role of Axn as the scaffold – In the absence of Wnt, GSK-3 phosphorylates Axn, promoting an 'open' conformation that allows Axn to bind β-catenin, as well leaves it available to interact with LRP5/6 in the event of Wnt stimulation. Following Wnt stimulation, Axn is dephosphorylated by PP1γ, thus decreasing its affinity for LRP5/6 as well as for β-catenin. This dephosphorylated Axin undergoes an intra-molecular conformation change by which its DIX domain interacts with the BCD domain, thus rendering it unavailable for interaction with either complex. The authors suggest that the closed conformation of Axn promotes its dissociation from the LRP receptor complex, thus allowing LRP to bind more Axin, thus promoting activation of the Wnt pathway in a catalytic manner. On the other hand, the closed-open conformational change of Axn could also act as a sensor for β-catenin levels in the cells, by promoting open conformation in the presence of excess β-catenin, thus regulating its levels very tightly (Kim, Huang et al. 2013). Although more work is needed to address how the dephosphorylation of Axn at separate residues affects its role in the two complexes, these findings have uncovered an exciting new role for Axn as not just the scaffold protein but also as an active mediator of the switching between pathway activation states.

Adenomatous Polyposis Coli (APC)

APC tumor suppressor gene has two Drosophila homologs – Dapc1/DApc2, that are ubiquitously expressed and act as functionally redundant proteins throughout development, except in the fly retina where DApc2 levels are reported to be very low, such that DApc1 mediates all the Wg transduction. Mutations in D-APC result in inappropriate activation of the Wg signaling pathway.

In the degradation complex, APC interacts with Axin via the SAMP repeats, and with Arm via the Arm-binding-repeats (seven 20 amino acid repeats and three 15 amino acid repeats). APC has been reported to have a dual role in the regulation of Arm – binding and sequestering cytoplasmic Arm; and escorting the phosphorylated Arm to the proteasome for degradation (Kimelman and Xu 2006, Lu, Lin et al. 2011). APC has also been reported to act in the nucleus to inhibit Wg signaling by favoring the formation of co-repressor complexes at Wg target genes (Henderson 2000, Sierra, Yoshida et al. 2006) and by binding Arm with higher affinity than Pan to export it from the nucleus (Rosin-Arbesfeld, Townsley et al. 2000).

In addition to its function as a negative regulator of Wg signaling, APC has also been reported to cause activation of Wg signaling at low levels. This activity has been attributed to regulation of Axn levels by DApc1/DApc2 (Benchabane, Hughes et al. 2008, Takacs, Baird et al. 2008).

Shaggy (Sgg) – Glycogen Synthase Kinase-3

Glycogen Synthase Kinase – 3 (GSK-3) is a ubiquitously expressed evolutionarily conserved protein kinase that was originally identified as an enzyme capable of phosphorylating and inactivating Glycogen synthase enzyme (Rylatt, Aitken et al. 1980). The Drosophila homolog is reported to be the early embryo patterning gene *shaggy/zeste-white 3* (Bourouis, Moore et al. 1990, Siegfried, Perkins et al. 1990). It was later shown to play a role in many different regulatory processes, including the Wg signaling pathway. GSK-3 interacts with its substrates via two sites – the priming phosphate site, and the active site. In the Wg signal transduction pathway, CK1α causes the priming phosphorylation of Arm and Axn. This aligns the substrate appropriately in the catalytic site for further phosphorylation by GSK-3. These substrates are then phosphorylated by Sgg (GSK-3) at multiple residues.

Sgg plays a dual role in Wg signal transduction – phosphorylation of Arm targets it for degradation, thus inhibiting the pathway activation. On the other hand, it also phosphorylates Arrow in the signaling complex, thus promoting catalytic activation of the pathway (Zeng, Tamai et al. 2005).

The mechanism by which Wg signaling causes inhibition of Sgg is not very well understood. The two prevalent models are the blocking of GSK-3's priming site by pre-phosphorylated LRP5/6 versus the sequestration of GSK-3 in multivesicular bodies as a means of inhibition. Both these models are reviewed in (Metcalfe and Bienz 2011)

2.3.3 Wingless as a morphogen in *Drosophila* development

Wingless has been studied extensively as a candidate for a morphogen based on several features. It is a secreted protein known to affect patterning of neighboring cells. Effects of loss of transduction or ectopic activation of Wg signaling cause widespread tissue patterning phenotypes, even though the protein production is limited to a subset of cells in the tissue (Struhl and Basler 1993). Wg induced developmental patterning has been studied in many different tissues in *Drosophila*, including the patterning of the embryonic epidermis, limbs, brain, eyes and wings. An example of Wg as a short range patterning molecule has been demonstrated in the patterning of the denticle belts in the embryo. *wg* gene is expressed in a single row of embryonic epidermal cells in each segment, but the movement of Wg across the neighboring rows of cells generates two distinct epidermal fates: a trapezoidal belt of hook-shaped denticles is produced on the ventral surface by six rows of epidermal cells in each abdominal segment (Baker 1988), reviewed in (Bejsovec 2013). Each row of denticles within a belt has a characteristic size, shape and polarity. These denticle belts are separated by smooth expanse of cuticle secreted by the more posterior rows of cells in each segment. Loss of *wg* transduction results in loss of the naked cuticle region, as well loss of the distinctive denticle morphologies. Conversely uniform ectopic activation of the *wg* signaling pathway leads to the conversion of the entire ventral epidermis to the naked cuticle fate, reviewed in (Bejsovec 2013).

A classic example of Wg as a morphogen acting over long range was demonstrated in the growth and patterning of the wing. Wing disc begins as an invagination of about 50 cells

at the time of hatching. Over the course of larval development, the cells undergo exponential proliferation, and the wing imaginal disc contains about 50,000 cells at the end of the third larval instar (Phillips, Roberts et al. 1990). Wg is expressed in a dynamic pattern throughout wing development: it is first detected in about 10 cells of the wing imaginal disc at the beginning of the second larval instar (Williams, Paddock et al. 1993). By the end of the second larval instar, it is expressed in the entire ventral region of the disc. This expression pattern is opposed by the expression of *apterous* (*ap*) in the dorsal portion of the second larval instar wing disc. These gene expressions lead to the D/V patterning of the wing (Garcia-Bellido, Ripoll et al. 1976). In *wg* mutant wing discs, *ap* expression expands throughout the wing disc, suggesting that Wg acts to restrict the *ap* expression domain (Williams, Paddock et al. 1993).

By the third larval instar, *wg* expression is constrained to a narrow strip of cells at the presumptive wing margin, along the D/V border by Notch signaling (de Celis, Garcia-Bellido et al. 1996). Wingless signaling at the boundary induces the expression of *delta* (*dl*) and *serrate* (*ser*), ligands of Notch, thus setting up a positive feedback mechanism for maintaining expression of Notch and Wg at the boundary cells (Diaz-Benjumea and Cohen 1995, Rulifson, Micchelli et al. 1996). Although Wg expression is restricted to a narrow strip of cells at the D/V, all the cells of the wing pouch experience Wg signaling. Wg protein is observed in a steep gradient up to 10-15 cell diameters away from the source, and target genes are expressed in a concentration dependent manner (Zecca, Basler et al. 1996, Neumann and Cohen 1997).

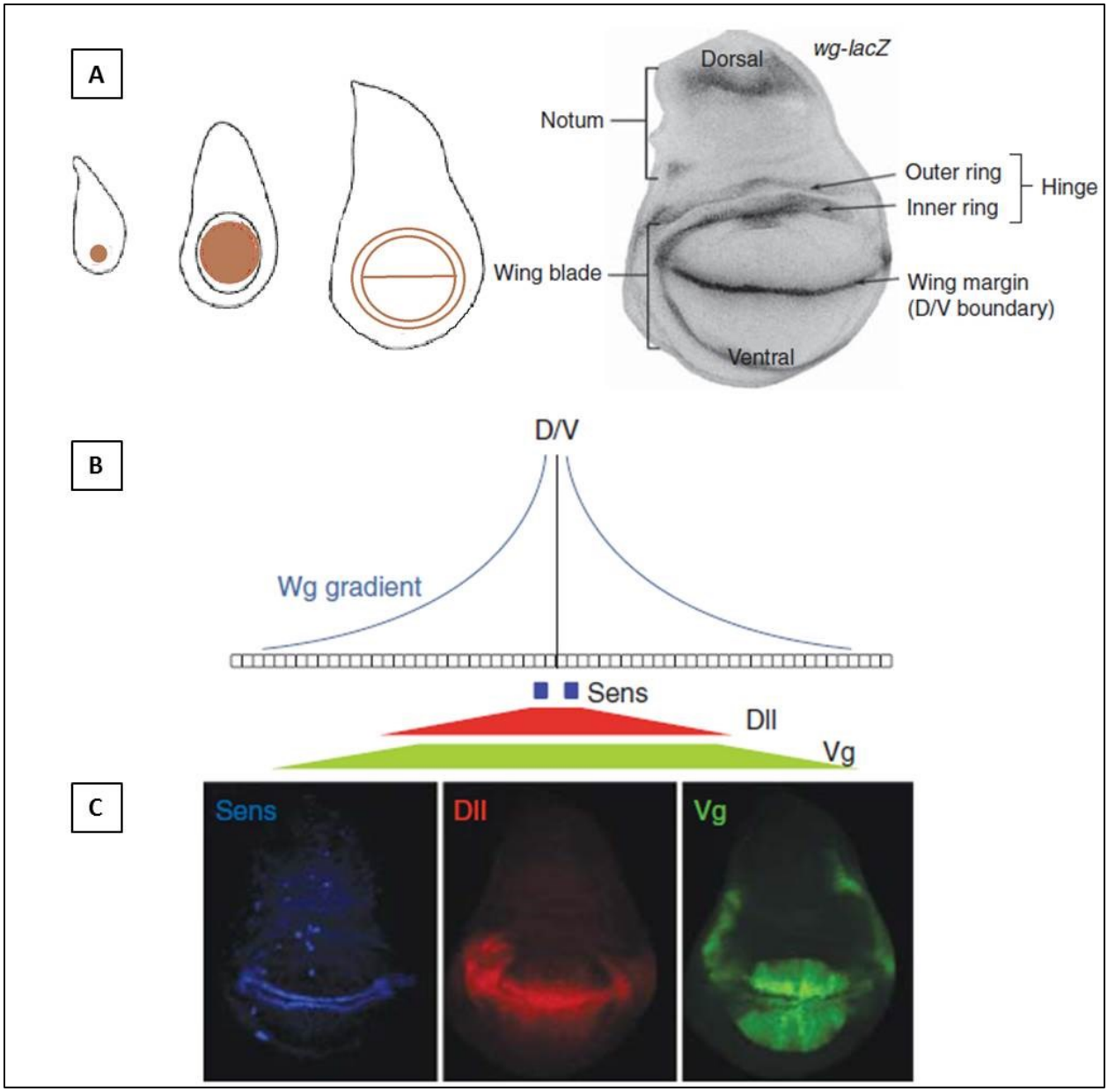

Figure 7: Wingless acts as a morphogen to pattern the wing disc along the D/V axis

A: Cartoon representation of Wg expression in the 1st, 2nd and 3rd instar larval wing discs.

The image shows *Wg-lacZ* expression, indicating the domains of Wg expression. Also highlighted are the adult structures which form from these domains.

B: Cartoon representation of the Wg gradient at the D/V border, and the threshold dependent expression of the target genes *senseless (sens), distalless (dll)* and *vestigial (vg)*.

C: Expression domains of the three target genes in the wing imaginal disc.

Images taken from (Swarup and Verheyen 2012)

In response to high levels of Wg signaling, the cells adjacent to the D/V border express *neuralized* (*neur*) and *senseless* (*sens*); which are responsible for the formation of specialized bristles at the adult wing margin (Couso, Bishop et al. 1994). Lower threshold targets *distalless* (*dll*) and *vestigial* (*vg*), responsible for wing blade proliferation, are expressed in a graded manner in the wing blade, with the levels gradually decreasing away from the D/V border (Zecca, Basler et al. 1996, Neumann and Cohen 1997). The various experiments conducted to classify Wingless as a morphogen in the patterning of the wing are as follows: Clones of cells expressing ectopic Wg caused the expression of *vg* and *dll* up to 10 cell diameters away, and up regulated *Dll-lacZ* up to 5 cell diameters away (Zecca, Basler et al. 1996). *Neur-lacZ* expression was observed in the cells immediately surrounding the clones. This is consistent with the activation of target genes in a concentration dependent manner.

Clones expressing a membrane-tethered form of Wg (*Nrt-Wg*) could activate the expression of the target genes only in the cells immediately adjacent to the clones. Meanwhile, clones expressing a mutant version of Arm (which lacks the N-terminal phosphorylation region, thus causes cell autonomous Wg signaling activation) activate

target gene expression only in the cells within the clone (Zecca, Basler et al. 1996). These results suggest that Wg is directly responsible for patterning of the wing, not via any signal relay mechanism.

Clones of cell mutant for *arm* show a concomitant loss of *vg* and *dll* expression. They stop dividing and are actively eliminated from the wing epithelium. This indicates that Wg signaling is continually required in the cells to maintain target gene expression (Zecca, Basler et al. 1996). Although these experiments show that Wg does act at long range to influence patterning, it acts to sustain *vg* and *dll* expression rather than initiate their expression, as would be expected from a classical morphogen. It was also shown that initial *vg* expression in the presumptive wing blade also depends on the Dpp signal emanating from the anterior compartment cells along the A/P border (Kim, Sebring et al. 1996). This *vg* zone was shown to broaden gradually in a manner correlating with cell proliferation, thus challenging the simple Wg diffusion induced activation theory (Kim, Sebring et al. 1996). Later work showed that Wg activated *vg* expression in the pouch via the Quadrant Enhancer (QE) element (Williams, Paddock et al. 1994, Kim, Sebring et al. 1996). However, as previously mentioned, Wg alone was insufficient to activate *vg* expression at a distance from the D/V border. Instead, it has been reported to act in concert with a short-range signal produced by the cells already expressing *vg*. This combinatorial signaling input is believed to act in a 'feed-forward' loop mechanism to add cells to the growing wing primordium in response to Wg signaling (Zecca and Struhl 2007, Zecca and Struhl 2007).

Although the role of Wg as a morphogen has been elucidated in such detail in the developing wing disc, we shall be utilizing the developing eye disc to study a Wg gradient. This Wg gradient patterns the periphery of the retina, and produces concentration dependent molecular responses that are associated with distinct adult morphological features. Thus it would be interesting to follow the gradient interpretation process at the molecular as well as cytological levels.

3 The eye of the fly – structure and formation of the *Drosophila* retina

3.1 The structural features of a *Drosophila* retina

The neuro-crystalline lattice presented by the Drosophila compound eye has fascinated researchers for a long time. The adult fly eye is made from approximately 800 units called ommatidia, each of which contains eight photoreceptors (R1-R8 cells), four cone cells overlying the photoreceptors and two 1º pigment cells. The ommatidia are positioned in a hexagonal array composed of secondary and tertiary pigment cells (2º/ 3º pcs) (Ready, Hanson et al. 1976, Wolff and Ready 1991). Standard ommatidia also project a mechanosensory bristle above the surface of the eye.

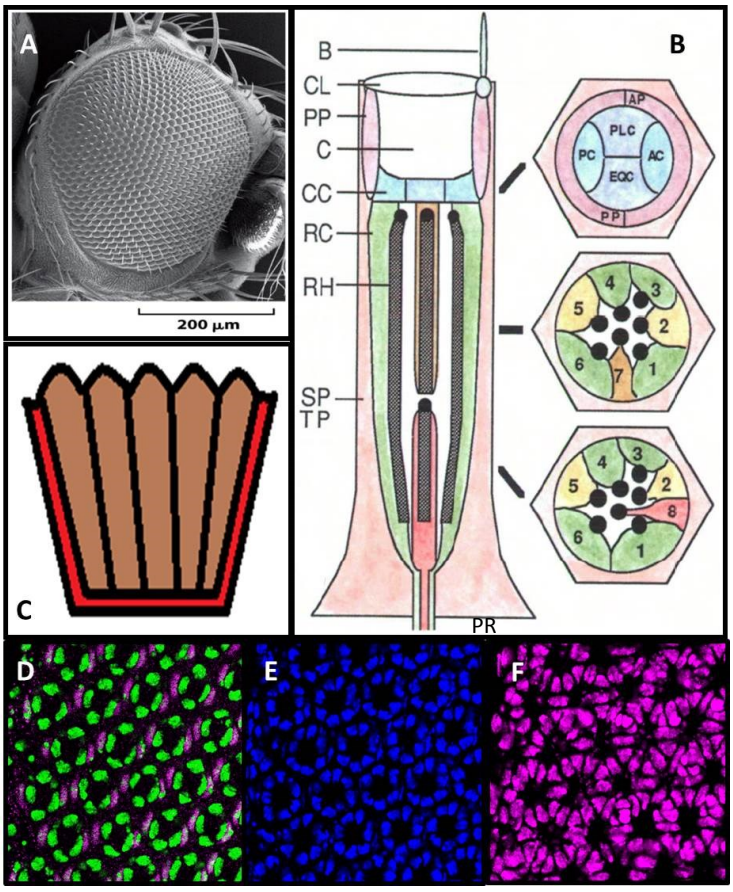

Figure 8: Structure of the ommatidium

A: Electron micrograph of the adult eye. B. Schematic of the organization of cells within an ommatidium. B-bristle, CL – corneal lens, C-cone, PP-1° pigment cell, CC-cone cell, RH – rhabdomere, RC – photoreceptor cells, SP - 2° pigment cell, TP - 3° pigment cell, PR- pigment rim C: The ommatidia are arranged in a niche of pigment cells. D, E, F: Pupal eye immunostaining at different retinal depths. D: At apical levels, we see cone cells (Green) and PP (in pink). E: In the middle we see photoreceptors (in blue). F: More basal sections show SP/TP pigment lattice (in pink). Figure adapted from (TOMLINSON 1988)

These cell subtypes within an ommatidium are specialized to perform various functions as described below:

a. The corneal lens – The surface of each ommatidium is covered by a hexagonal extracellular secretion from the cone cells and pigment cells, referred to as the lens. Underneath the lens, a clear, gel-like substance (the pseudo cone) is secreted by the cone cells, which acts as the second refractile element.
b. Cone cells – There are 4 cone cells, with their nuclei arranged just beneath the pseudo cone, with the equatorial and polar cone cells contacting each other in the center. These cone cells extend thin projections in between the photoreceptor cells, all the way to the base of the retina, where these projections end in sac-like structures referred to as the ‘cone-cell feet’. These sacs are filled with ommachrome pigment granules. At the base, the contacts between the cone cells

are reversed. Now, the anterior and posterior cone cells are in contact. The primary purpose of the cone cells is to secrete the pseudo cone, which aids in refracting light onto the photoreceptors; and to maintain the structural integrity of the ommatidium.

c. Photoreceptors – These are the neuronal cells of the ommatidium, and there are 8 of these cells. Based on their precise arrangement within an ommatidium, individual photoreceptors are identified as R1-R8. R1-R6 photoreceptors, collectively termed as the 'outer photoreceptors' are arranged in a trapezoid manner. They have large rhabdomeres – the condensed apical microvillar membranes of the photoreceptor neurons that are rich in rhodopsin, and serve as the light transduction channels. These outer photoreceptors express rhodopsin Rh1, and are chiefly involved in motion detection. The two central photoreceptors R7/R8 are specialized photoreceptors. The rhabdomere of the R7 photoreceptor cell is present atop the rhabdomere of the R8 photoreceptor cell. Also, unlike the outer photoreceptor axons that project to the first layer of the optic lobe, the lamina; these inner photoreceptor axons project deeper into the medulla. The R8 axon terminates in layer M3, and the R7 axon terminates in M6 layer of the medulla. Correspondingly these axons are termed long visual fibers, whereas the outer photoreceptor axons are termed short visual fibers. Additionally, the inner photoreceptor cells express different rhodopsins, Rh3/Rh4 in R7 cells and Rh5/Rh6 in R8 cells. The different combinations of these rhodopsins provide UV and color vision capacity to the fly retinas. In addition to these specializations, a

subset of ommatidia has photoreceptors that are specialized for plane-polarized light detection, as discussed in detail later in this section.

d. Pigment cells – They consist of the primary (1°), secondary (2°) and tertiary (3°) pigment cells. The 1° pigment cells are the only cells which do not extend the depth of the retina, instead they are present in the apical region. The symmetrical anterior and posterior 1° pigment cells contain brown ommachrome granules and flank the cone cells and the pseudocone. The 2° and 3° pigment cells form the hexagonal lattice within which all the ommatidia are nestled. These pigment cells are shared between the ommatidia. They express both ommachrome and pteridine pigment granules, lending the rich red color characteristic of the Wild Type fruit fly eye. The apical tips of these cells are present just beneath the lenses and then extend all the way to the base of the retina, where these cells flatten out and form plates that act as the base of the retina. The axon fiber bundles from each ommatidium pass through the basement fenestrated membrane formed by these pigment cells in order to reach the optic lobe. In addition to the inter-ommatidial pigment cell lattice, a thick layer of pigment cells is found at the periphery of the eye, to be discussed in detail later.

e. Mechanosensory bristles – These are the second set of sensory cells in the retina. About 600 bristles project out from the surface of the retina, at every alternate vertex of the hexagonal array. Each bristle group comprises of 4 cells – the socket secreting tormogen, bristle secreting trichogen, the bristle cell and the supporting

glial cell thecogen. During pupal development the trichogen and tormogen degenerate, leaving only the other two cells in the adult.

3.2 Development of the eye

Based on several years of developmental studies, we have a lot of insight into the formation of the precise ommatidial pattern which is then repeated throughout the retina to form the hexagonal array. Ommatidial cells are not arranged in a lineage dependent manner, rather they are recruited form a set of equipotent cells based on their position and response to signals from the previously assembled ommatidial cells that enable them to occupy a niche, and then develop into a specialized cell type.

3.2.1 Early eye development

The primordial cells which eventually give rise to the adult visual system are set aside during cellular blastoderm stage of early embryogenesis (Simcox and Sang 1983). A group of 20 founder cells originating from the anterior dorsolateral region of the early embryo will give rise to the presumptive eye field including the larval eye, the eye antennal imaginal disc and the precursors of the adult optic lobes (Green, Hartenstein et al. 1993). The eye-antennal disc is made up of a columnar epithelial layer, called the Main Epithelium or the disc proper; and a squamous epithelial layer called the Peripodial Membrane (Haynie and Bryant 1986). The disc proper will form the adult antenna and the eye, while the peripodial membrane will give rise to the adult head capsule (Bessa and Casares 2005). The development of the eye-antennal disc begins at stage 17 of embryogenesis, when the cells of the presumptive disc are compressed into a pouch-like

structure. The inner cells of the pouch will give rise to the medial wall of the disc proper, while the outer cells will form the peripodial membrane (Pastor-Pareja, Grawe et al. 2004).

Formation of the presumptive eye disc is caused by the expression of the genes *eyeless (ey)* and *twin-of-eyeless (toy),* both of which have been shown to be required and sufficient to lead to eye-antennal cell fate determination (Czerny, Halder et al. 1999). These genes are the primary players of a complicated genetic circuit called the Retinal Determination Network (RDN). Other genes known to be involved in the RDN are *sine oculis*, *optix*, *dachshund, teashirt*, *eyegone*, *twin-of-eyegone*, *eyes absent*, *distal antenna*, *distal antenna-related* and *tiptop*. All these genes possess the ability to induce ectopic eye formation upon being overexpressed (although the potential of the individual gene to accomplish this varies). Mutational inactivation of these genes leads to eye development defects (Bui, Zimmerman et al. 2000, Zimmerman, Bui et al. 2000). The combinatorial action of these genes, in addition to many novel members of the RDN leads to the determination and specification of the eye-antennal imaginal disc (Michaut, Flister et al. 2003).

The cells of the eye-antennal imaginal disc continue to proliferate through the first larval instar stage. While the disc grows in size, the developmental plasticity of the cells is maintained until the second larval instar stage, as indicated by the fact that mitotic clones generated prior to the second instar may form a part of any of the structures formed by the eye-antennal disc (Morata and Lawrence 1979). During the second larval instar, *ey*

and *toy*, which were formerly expressed uniformly throughout the disc, now show expression restricted to the posterior region, which will ultimately form the eye disc region (Kammermeier, Leemans et al. 2001, Kumar and Moses 2001, Kenyon, Ranade et al. 2003). Similarly, *homothorax* (Hth), a transcription factor expressed uniformly throughout the disc during the first instar, now shows expression restricted to the anterior part of the disc, which will ultimately form the antennal disc region (Bessa, Gebelein et al. 2002). Hth maintains expression in the presumptive head capsule region, and also plays a role in later pupal differentiation events (Pai, Kuo et al. 1998). The homeobox gene *cut* is the first marker for the antenna, and is expressed solely in the presumptive antenna portion of the second instar disc. This expression is followed by the expression of *distalless*, and both these gene expressions have been shown to be required for the formation of the antenna (Bodmer, Barbel et al. 1987, Dong, Chu et al. 2000, Kenyon, Ranade et al. 2003). Once segregated thus, the eye and antennal precursor fields are maintained by mutual repression of the marker genes – Cut and Hth repress *ey* transcription in the antennal domain, while So represses *cut* and *hth* expression in the presumptive eye field (Wang and Sun 2012). The antennal disc will give rise to the adult antenna and the head capsule, whereas the eye disc will give rise to the adult eye proper, head capsule and the ocelli (Haynie and Bryant 1986).

The final lockdown on the competence of eye disc precursor cells for retinal differentiation occurs via the delayed co-expression of a subset of the RDN genes, referred to as the *Early Retinal Genes*, namely *eya*, *so* and *dac* (Desplan 1997, Kumar and Moses 2001, Kenyon, Ranade et al. 2003). The expression of these genes in response

to the interactions of the extracellular signaling pathways, leads to the determination and proliferation of the presumptive eye field (Kumar and Moses 2001, Baker and Firth 2011).

wg and *dpp* are expressed in opposing domains in the early second larval eye disc, with *wg* being expressed along the anterior dorsal end and *dpp* being expressed along the posterior dorsal end of the eye disc (Cho, Chern et al. 2000). Wg acts as a suppressor of eye development by antagonizing Dpp (Hazelett, Bourouis et al. 1998). Ectopic expression of Wg leads to the abolishment of early retinal gene expression; while loss of Wg signaling in the eye disc causes ectopic expression of *so, eya,* and *dac* (Baonza and Freeman 2002). Dpp acts as a promoter of eye development. Ectopic eye formation by expressing RDN genes have been reported to show spatial restriction to *dpp* expressing domains (Chen, Halder et al. 1999, Salzer, Elias et al. 2010). Dpp is required for the initiation of early retinal gene expression, but it is not essential for maintaining their expression (Curtiss and Mlodzik 2000). Also, loss of Dpp signaling in the eye disc leads to reduction in *so*, *eya* and *dac* expression (Chen, Halder et al. 1999).

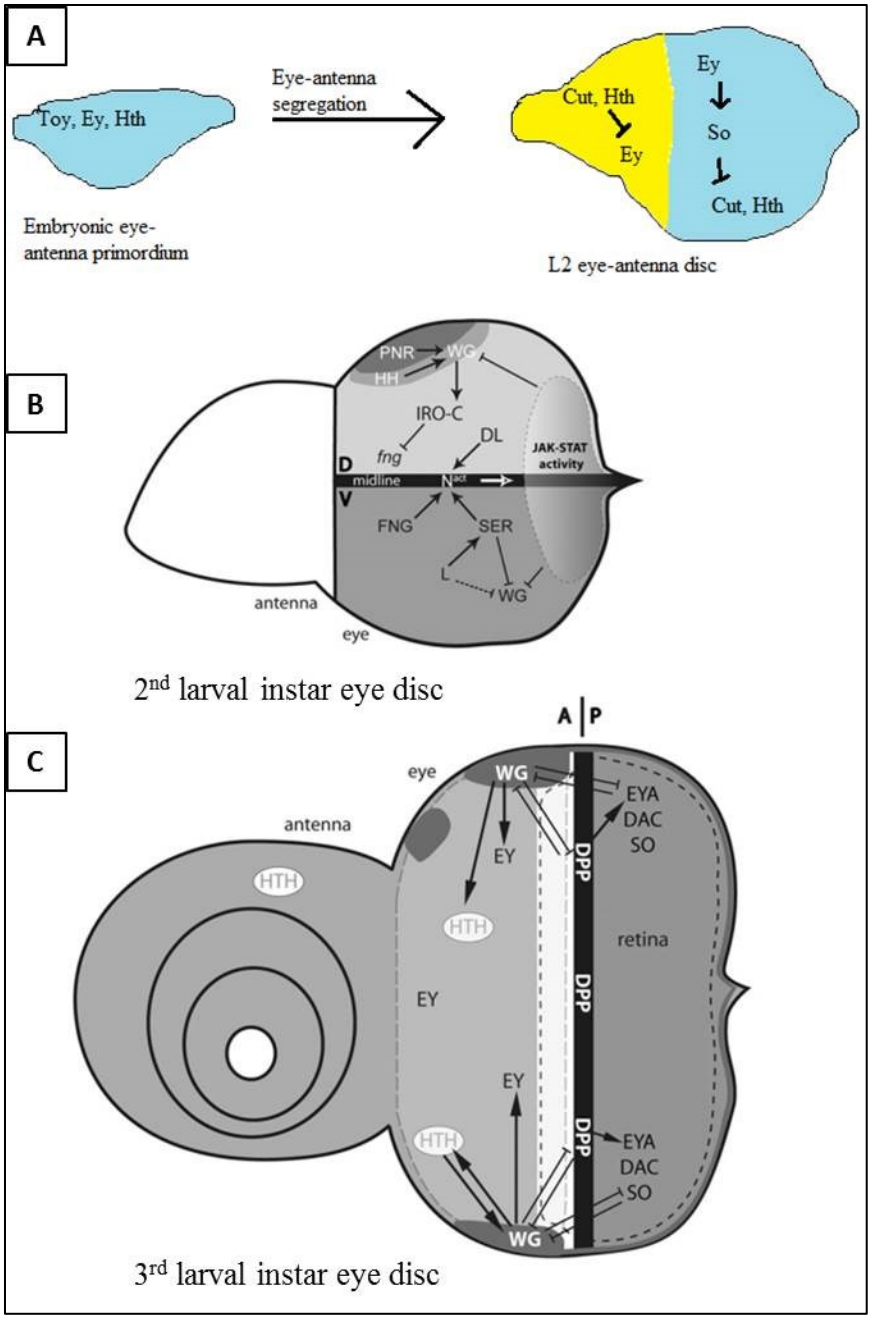

Figure 9: Summary of signaling events in the developing eye disc

A: In the early second instar disc, eye antennal fields are segregated by the mutually repressive actions of Ey and So.

B: In the second larval instar eye disc, the D/V axis of the retina is specified by the concerted action of L, Ser, Wg and the Iro-C proteins.

C: In the third larval instar, differentiation of the eye disc proceeds by the formation of the morphogenetic furrow. The initiation and progression of the furrow requires the activity of Wg, Dpp and Hh signaling pathways.

Images B and C taken from (Roignant and Treisman 2009)

Once these opposing domains have been set up, a third input is required to skew the balance towards eye field formation versus head capsule formation. This input is provided by the Notch signaling pathway. The Notch receptor is activated along the dorso-ventral margin (also known as the signaling center of the developing eye) (Cho and Choi 1998) of the eye by opposing expression domains of its ligands Delta (Dl) and Serrate (Ser) (Kenyon, Ranade et al. 2003). Activation of the Notch signaling pathway leads to the expression of *eyegone (eyg) (Dominguez, Ferres-Marco et al. 2004)* which in turn activates the expression of *unpaired (upd)*. Upd is a ligand of the Jak/Stat signaling pathway and as it is secreted, it promotes growth and proliferation of the cells of the eye disc (Chao, Tsai et al. 2004). The model proposed for determination of the eye field is thus: Notch signaling promotes growth of the entire eye disc, leading to the separation of the Wg and Dpp expressing domains. The cells which no longer sense Wg, but sense Dpp in the more posterior regions now activate expression of *so*, *eya* and *dac*; thus initiating eye field formation (Kenyon, Ranade et al. 2003). Although this model does not fully explain the restriction of the marker genes to the eye and antennal domains prior to Wg

and Dpp interaction, it supports an indirect role of Notch signaling in the specification of the eye (Kumar and Moses 2001, Dominguez and Casares 2005).

Unlike the wing and leg imaginal discs, the eye disc is not divided into lineage restricted compartments during early embryogenesis. The generation of the dorso-ventral (D/V) border during the second larval instar is the first instance of compartmentalization in the developing eye disc (Singh, Tare et al. 2012). The early eye disc is entirely ventral in fate, as indicated by the expression of ventral selector genes *Lobe (L)* and *Serrate (Ser)* throughout the early second instar eye disc (Dominguez and de Celis 1998, Papayannopoulos, Tomlinson et al. 1998, Singh and Choi 2003, Singh, Chan et al. 2005). The expression of *pannier (pnr)* along the dorsal margin of the eye leads to the subsequent restriction of L and Ser expression to the ventral half of the eye (Singh and Choi 2003, Singh, Tare et al. 2012). Thus the dorsal fate is superimposed on the eye disc cells. *wg* acts downstream of *pnr* to promote the expression of the *Iroquois complex (Iro-C)* genes in the dorsal half of the eye (Treisman and Heberlein 1998, Cavodeassi, Diez Del Corral et al. 1999, Maurel-Zaffran and Treisman 2000). The Iro-C proteins restrict the expression of *fringe (fng)*, a glycosyl transferase which modulates the interaction of Notch with its ligands Dl and Ser (Cho and Choi 1998, Dominguez and de Celis 1998, Cavodeassi, Diez Del Corral et al. 1999, Bruckner, Perez et al. 2000). The restriction of *fng* to the ventral domain, along with expression of Dl and Ser in opposing domains leads to the activation of the Notch pathway at the D/V midline (Cho and Choi 1998, Dominguez and de Celis 1998, Papayannopoulos, Tomlinson et al. 1998). The compartment border between the dorsal and ventral compartments thus generated is

maintained by antagonistic interactions between the genes of either compartment (Singh, Chan et al. 2005). The eye primordium formed thus is now competent to undergo differentiation and ultimately form the adult retina.

3.2.2 The morphogenetic furrow

The onset of differentiation of the retinal precursor cells occurs in the third larval instar, and is marked by the formation of a morphogenetic furrow (Ready, Hanson et al. 1976) at the intersection of the D/V midline and the posterior margin of the eye field (Hsiung and Moses 2002, Treisman and Lang 2002). The differentiating cells undergo apical constriction and apico-basal contraction, thus causing the formation of a groove on the surface of the epithelium, hence the name (Tomlinson and Ready 1987, Wolff and Ready 1991). The furrow is initiated at the posterior margin, and it proceeds anteriorly in a wave fashion, with proliferating undifferentiated cells present ahead of the furrow, and rows of clusters of differentiated cells arising posterior to the furrow. About 30 rows will lead to the formation of the entire retina. As it takes approximately 90 minutes for one row formation, the journey of the morphogenetic furrow across the entire eye disc takes about 2 days (Ready, Hanson et al. 1976, Strausfeld and Campos-Ortega 1977, Tomlinson and Ready 1987).

The initiation of the furrow at the precise intersection of the D/V midline and the posterior margin requires the interaction of *hedgehog (hh)*, *dpp* and *wg*. Prior to furrow initiation, Hh and Dpp are expressed at the posterior margin of the eye. However just before initiation, Hh expression coincides with the posterior center, while Dpp

expression, although present at the lateral margins, is absent at the center (Masucci, Miltenberger et al. 1990, Raftery, Sanicola et al. 1991, Dominguez and Hafen 1997, Borod and Heberlein 1998). Loss of either one of these genes results in loss of endogenous furrow formation, while ectopic expression induces ectopic furrows (Chanut and Heberlein 1995, Ma and Moses 1995, Strutt, Wiersdorff et al. 1995, Treisman and Rubin 1995, Wehrli and Tomlinson 1995, Wiersdorff, Lecuit et al. 1996, Dominguez and Hafen 1997, Pignoni and Zipursky 1997, Borod and Heberlein 1998). Wg is expressed along the lateral margins of the disc, and serves to ensure the initiation of the furrow precisely at the posterior center. Loss of *wg* signaling induces ectopic furrow formation, while ectopic expression of Wg in clones prevents furrow progression (Ma and Moses 1995, Treisman and Rubin 1995). Wg expression is repressed at the posterior center in order to accomplish furrow initiation. This repression is achieved by the Jak/Stat pathway ligand Upd, which is expressed exclusively at the posterior center prior to furrow initiation (Pignoni and Zipursky 1997, Chao, Tsai et al. 2004, Tsai and Sun 2004). The temporal controls of furrow initiation are not fully understood. Possible hypotheses include triggering via ecdysone (Niwa, Hiromi et al. 2004), and loss of restrictive signaling from the anterior portion owing to growth of the eye disc (Ma and Moses 1995, Treisman and Rubin 1995, Kenyon, Ranade et al. 2003).

Although furrow initiation is a unique developmental event, progression of the furrow occurs via repeated signaling events driven by an auto regulatory feedback loop. Prior to entering the furrow, the cells anterior to the furrow undergo a cell-cycle arrest at G1 phase (Wolff and Ready 1991, Thomas, Gunning et al. 1994). Upon exiting the furrow,

some cells exit the cell cycle and begin differentiation as photoreceptors, while the remaining unspecified cells undergo one more round of mitotic division (the second mitotic wave) to generate precursors for the differentiation and assembly of the entire ommatidial array (Wolff and Ready 1991). The G1 arrest is essential to co-ordinate a synchronous exit of the differentiating clusters from the entire length of the furrow, thus marking the beginning of the patterning of the retina. Furrow progression is regulated by multiple signaling molecules. Hh produced by the differentiated cells behind the furrow induce the anterior undifferentiated cells to enter the cell cycle arrest and undergo differentiation. These cells, upon differentiation will produce Hh which will signal the further anterior cells to enter the furrow, thus setting up an auto regulatory mechanism of furrow progression (Blackman, Sanicola et al. 1991, Heberlein, Wolff et al. 1993, Treisman and Heberlein 1998, Greenwood and Struhl 1999).

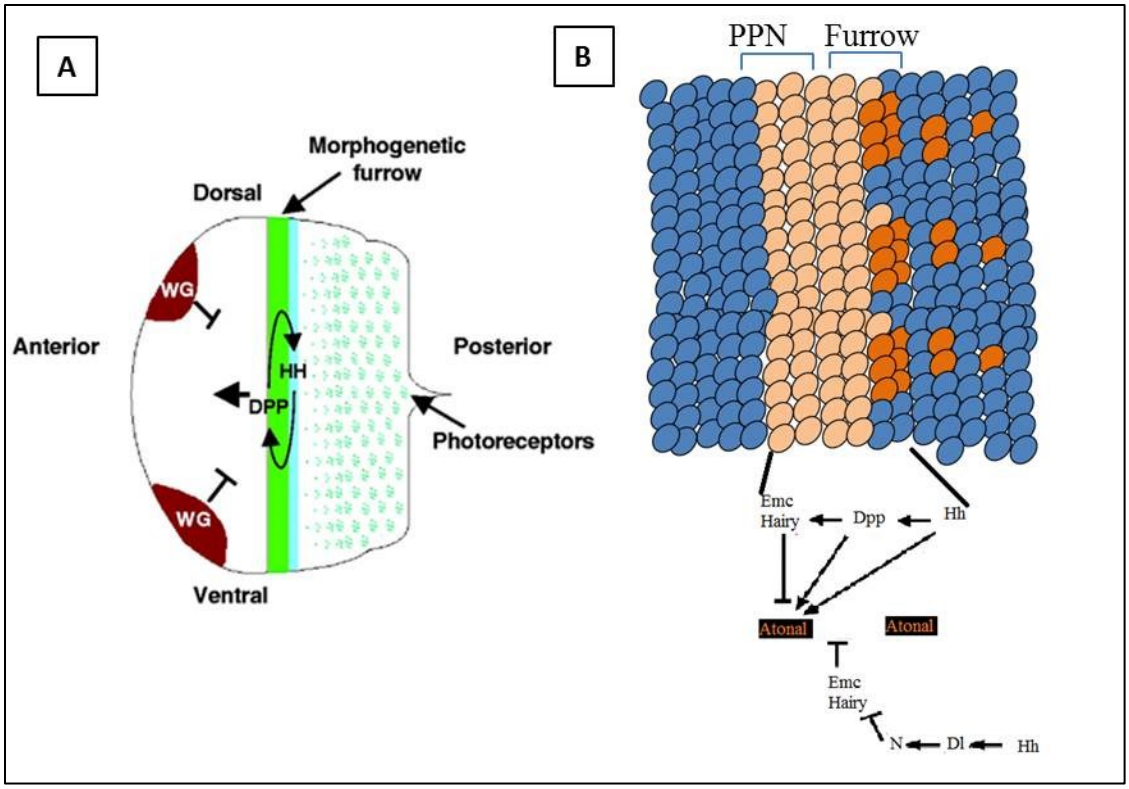

Figure 10: Progression of the morphogenetic furrow in an auto regulatory feedback loop

Cartoon depicts the signaling events at the morphogenetic furrow that allow furrow progression. The Hh-Dpp feedback induction loop allows the next row of anterior undifferentiated cells to enter the PreProneural (PPN) state, following which they undergo differentiation. The cartoon on the right depicts the signaling events that lead to the Atonal expression in the furrow, following by pruning of the expression to one cell per cluster. Image adapted from (Freeman, 2007).

The cells entering the furrow also activate *dpp* transcription. Dpp is expressed in and posterior to the furrow, and serves two functions: it co-ordinates cell cycle synchronization of the cells anterior to the furrow, and it antagonizes Wg diffusing in

from the lateral margins, thus allowing furrow progression (Burke and Basler 1996, Wiersdorff, Lecuit et al. 1996, Horsfield, Penton et al. 1998, Greenwood and Struhl 1999, Curtiss and Mlodzik 2000, Firth, Bhattacharya et al. 2010). The cell cycle synchronization of the cells via long range action of Dpp induces the cells to enter a 'pre-proneural state' – marked by the expression of the genes *hairy (h)* and *extramacrochaetae (emc)* (Greenwood and Struhl 1999, Baonza and Freeman 2001). H is a bHLH (basic helix-loop-helix) DNA binding protein while Emc is an HLH transcription factor that regulates transcription by sequestering other bHLH transcription factors away from their target domains. These proteins are expressed in a stripe just anterior to the furrow, and serve to slow down the furrow progression. Within the furrow, Hh induces Dl expression in the differentiating cells, which then induces Notch expression in the neighboring cells. Notch signaling pathway activation leads to expression of *atonal (ato)*, a proneural transcription factor, whose levels are maintained at low levels via H and Emc mediated repression (Brown, Sattler et al. 1995, Baonza and Freeman 2001). Upon overcoming this repression in response to Notch signaling, Ato is expressed in stripe at the edge of the furrow (Jarman, Grell et al. 1994, Jarman, Sun et al. 1995, Dokucu, Zipursky et al. 1996). Within the furrow, this expression is refined to evenly spaced clusters of 4-5 cells. As the clusters exit the furrow, only 2-3 cells within each cluster express Ato, and this cluster is now referred to as the 'R8 equivalence group'. From this group, eventually only one cell will retain Ato expression. This pruning is mediated by the Notch signaling pathway in combination with the transcription factors Rough and

Senseless (Cagan and Ready 1989, Jarman, Grell et al. 1994, Jarman, Sun et al. 1995, Baker, Yu et al. 1996, Dokucu, Zipursky et al. 1996, Chanut, Luk et al. 2000).

3.2.3 Assembling the ommatidia

The first precursors of ommatidial clusters emerge from the furrow as an arc of ~9 cells which then zipper shut to form a precluster of 6-7 cells, which includes the precursors to R2-5 and R8, and a couple of mystery cells which are later ejected. The Ato expression in the R8 equivalence group has now been narrowed down to one cell by Notch mediated lateral inhibition (Baker, Mlodzik et al. 1990, Baker, Yu et al. 1996, Baker and Yu 1997, Li and Baker 2001). This single cell will become R8, and it is the founder cell of the ommatidium. The subsequent cells are specified via sequential signaling and accretion. Thus ommatidial assembly is lineage independent (Ready, Hanson et al. 1976, Tomlinson 1985, Tomlinson and Ready 1987, Wolff and Ready 1991). The R8 cell now expresses Senseless and Rhomboid-1, which processes Spitz, a peptide which acts as the ligand for the Drosophila EGF Receptor (DER) (Dokucu, Zipursky et al. 1996, Baonza and Freeman 2001, Pepple, Atkins et al. 2008). EGF signaling to the cells adjacent to the R8 cell promotes Rough expression, which in turn represses R8 fate (Dominguez, Wasserman et al. 1998, Hayashi and Saigo 2001). These cells now become the R2/5 cells, and start expressing Spitz. This EGF signal is received by the abutting cells in the precluster, thus specifying them as R3/4 photoreceptors (Freeman 1996, Flores, Daga et al. 1998, Roignant and Treisman 2009). Once the 5 cell precluster is specified, the remaining undifferentiated cells undergo one more round of mitosis.

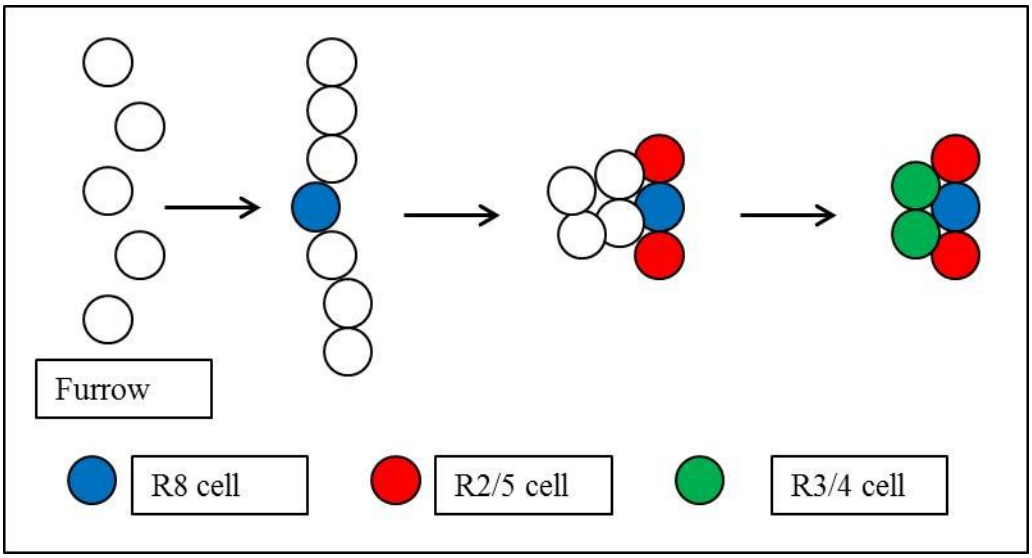

Figure 11: Schematic representation of the sequential specification of cells in the precluster to form the 5 cell precluster.

The R8 is the first photoreceptor to be specified followed by R2/5 and R3/4. This specification depends on EGFR signaling.

From this fresh pool of cells (referred to as second wave cells), 3 cells are added to the R2/8/5 side of the ommatidium. The cells adjacent to R2/5 undergo rapid differentiation to form the R1/6 cells, while the cell in the middle undergoes differentiation several hours later to form the R7 cell.

The Receptor Tyrosine Kinase (RTK) pathway and the Notch signaling pathway are utilized in the specification of R1/6/7 cells (Tomlinson 1985, Tomlinson and Ready 1987, Tomlinson 1989, Tomlinson and Struhl 2001, Tomlinson, Mavromatakis et al. 2011). RTK pathway activation leads to the transcription of phyllopod (*phyl*), which encodes an adaptor protein (Chang, Solomon et al. 1995, Dickson, Dominguez et al. 1995). Phyl

brings together Sina (a ubiquitin ligase) and another protein called Ebi, to lead to the polyubiquitination and subsequent degradation of Tramtrack (Ttk) via the proteasome pathway. Ttk is a transcription factor which represses photoreceptor differentiation. Notch activation prevents Ttk degradation, thus preventing photoreceptor differentiation. Of the second-wave cells added to the 5-cell precluster, the ones that manage to degrade Ttk become photoreceptors, while the cells that fail to degrade Ttk form the cone cells of the ommatidia (Li, Li et al. 1997, Tang, Neufeld et al. 1997, Li, Xu et al. 2002). The specification of R1/6/7 photoreceptors requires the action of two RTKs – DER and Sevenless (Sev). DER is expressed ubiquitously, and is required for the specification of the photoreceptors R1-6 (Freeman 1996, Kumar, Tio et al. 1998). Sev is expressed at high levels in the R3/4, R7 and in the cone cell precursor cells, and at low levels in the R1/6 precursor cells (Tomlinson and Ready 1986, Hafen, Basler et al. 1987). The presence of Sev in the R7 precursor cell subsequently allows R7 fate specification. The ligand for Sev RTK is Bride-of-Sevenless (Boss); a membrane bound peptide presented on the surface of R8 cell (Tomlinson, Bowtell et al. 1987, Reinke and Zipursky 1988). The R1/7/6 precursors contact R8 but the cone cell precursors do not. It was demonstrated later that the R 1/7/6 and the cone cells are part of an equivalence group, and the developmental fates of these cells are changeable upon manipulation of the Notch and RTK pathways (Tomlinson, Mavromatakis et al. 2011).

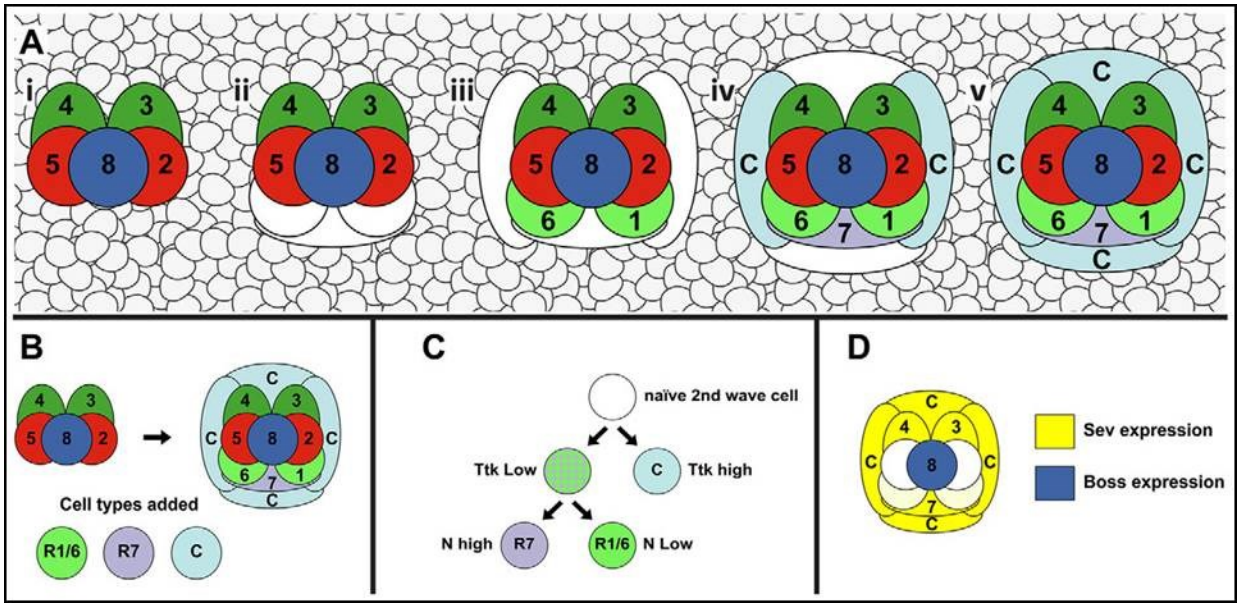

Figure 12: Schematic representation of the sequential addition of second wave cells to the 5-cell precluster, and the gene expression pattern involved in the process.

A: The incorporation and differentiation of the first seven cells to be added to the precluster. (i) The precluster (R2, 3, 4, 5, 8), is surrounded by a 'sea' of undifferentiated second wave cells (gray ovals). (ii) Three cells from the pool join the precluster on the R2/5/8 face. (iii) Two cells begin to differentiate as R1/6 photoreceptors while the R7 precursor between them delays differentiation and two cone cell precursors (C) join the cluster at the flanks. (iv) The R7 precursor begins to differentiate and two additional cone cell precursors join the cluster. (v) The differentiation of the cone cells ends this phase of ommatidial development with all seven of the newly added cells differentiating as specific cell types.

B: The three different cell types (R1/6, R7 and cone cells) that are added to the precluster.

C: The cell fate code for the R1/6, R7 and cone cells. If a cell degrades Ttk and has high N activity it becomes an R7, but if it has low N activity it becomes an R1/6 type. If the cell fails to degrade Ttk it becomes a cone cell.

D: The expression patterns of Sev and its ligand Boss.

Image taken from (Mavromatakis and Tomlinson 2013)

The proposed model for the specification of the R1/6, R7 and cone cells is as follows: The precluster cells express Spitz, as well as low levels of Dl. These low levels of Dl cause weak activation of Notch signaling in the three cells which join the precluster, thus providing weak repression to photoreceptor differentiation (Cooper and Bray 2000, Tomlinson and Struhl 2001). The cells adjacent to R2/5 receive the Spitz signal, activate DER and are able to overcome the Notch block, and differentiate as R1/6 cells. As they differentiate, these R1/6 cells express Dl at high levels (Tsuda, Nagaraj et al. 2002, Miller, Lyons et al. 2009). By this time, the flanking cone cell precursors have also occupied the anterior and posterior niches. The R7 precursor cell and these 2 cone cell precursors receive Dl signal at high levels from R1/6 cells, thus activating Notch to higher levels and preventing photoreceptor differentiation. In addition, high levels of Notch also lead to *sev* transcription. The interaction of Sev on the R7 precursor cell with its ligand Boss on the R8 cell provides high level activation of the RTK signaling pathway, thus enabling Ttk degradation and differentiation of the precursor cell as a photoreceptor. However, as the cell also has high levels of Notch, it differentiates as an R7 cell instead of R1/6, and starts expressing Dl at high levels. The flanking cone cells do

not contact R8 cell, hence they are unable to overcome the Notch mediated block on photoreceptor differentiation. These cone cells and the R3 also express Dl at high levels, thus causing high levels of Notch activation in the two cone cell precursors added later, and preventing their differentiation as photoreceptors. Thus the seven cells added to the precluster are equipotent, and their subsequent specification depends upon the niche they occupy which determines the developmental signals received by the precursor cells (Basler, Christen et al. 1991, Fortini, Rebay et al. 1993, Flores, Duan et al. 2000, Tomlinson and Struhl 2001, Tomlinson, Mavromatakis et al. 2011).

Following the cone cell additions, two primary pigment cells arise early on in pupal development and enwrap the cone cells, thus completing the ommatidial units (Waddington and Perry 1963, Cagan and Ready 1989, Cagan and Ready 1989). The interommatidial pigment cells (IPC) and the bristles are also specified in the first few hours of pupal development. The cells which manage to establish contacts with the 1°pigment cells form the 2°/3° pigment cells. This niche based recruitment depends on the interplay of Notch and EGFR signaling (Cagan and Ready 1989, Freeman 1996, Miller and Cagan 1998).

3.2.4 Pupal stages of eye development

During pupation, the cells of the ommatidia undergo terminal differentiation. The photoreceptors elongate their rhabdomeres, establish the appropriate axonal projections to the brain and begin expressing the rhodopsin genes. This results in the deepening of the retina and attaining the curvature of the adult eye (Cagan and Ready 1989). Once disc

eversion occurs, and by the time all ommatidial cell types are specified, spatially restricted apoptosis occurs throughout the retina in order to prune the pigment cell lattice. The undifferentiated cells which fail to establish any contacts with the 1° pigment cells get eliminated in this process (Cagan and Ready 1989). Interestingly, removal of either the 1° pigment cells or the cone cells via laser ablation experiments show increased apoptosis of the surrounding IPCs, suggesting that the ommatidial cells must be sending a 'survival' signal to the adjacent 2°/3° pigment cells (Miller and Cagan 1998). Genetic studies suggest that the life-and-death decisions for the IPCs depend on the interplay between Notch and EGFR signaling, with the Notch signal promoting apoptosis, and EGFR signaling aiding cell survival. Consistent with this hypothesis, Notch expression is restricted to the IPCs during mid pupal development (Kooh, Fehon et al. 1993). Spitz, a diffusible ligand of DER, is expressed in the cone cells and 1° pigment cells, and is suggested to be the survival signal (Miller and Cagan 1998).

Another important factor is the establishment and maintenance of cell-cell contacts that seems required to prevent apoptosis in the retinal lattice refinement process. Mutational analysis of the *Irregular chiasmC-roughest (IrreC-rst)* gene, which encodes a transmembrane protein with Immunoglobulin-like repeats, supports this hypothesis. IrreC-rst is known to be involved in cell adhesion, axon path finding, mediating cell movement and cell death (Wolff and Ready 1991, Reiter, Schimansky et al. 1996). Prior to the inter ommatidial apoptosis, the additional IPCs undergo extensive rearrangements into layers and the IrreC-rst protein accumulates at the borders between 1° pigment cells and IPCs in a Notch dependent manner (Grzeschik and Knust 2005). Loss of IrreC-rst

protein impairs this lattice reorganization and subsequent apoptosis (Bao and Cagan 2005). Elimination of the additional IPCs does not occur in a stochastic manner, rather there are higher levels of apoptosis near the equatorial and polar positions of each ommatidium, thus tightening the lattice into a more hexagonal array than a cuboidal one. This lattice refinement program continues at low levels for several hours through mid-pupation. Later pupation events include the tightening of the IPC lattice by the expression of the transcription factor Escargot (Lim and Tomlinson 2006), and the generation of pigment granules. The cone cells and the 1°pigment cells secrete the pseudocone and the corneal lens, which is continuous with the cuticle (Cagan and Ready 1989).

3.2.5 The structure and patterning of the fly eye periphery

In addition to the pupal development events described above, a series of specialized ommatidia develop at the periphery of the retina. These ommatidial rows are present immediately adjacent to the head capsule (HC) tissue; and the patterning occurs as a result of diffusing Wg signaling from the HC (Ready, Hanson et al. 1976, Tomlinson 2003). The peripheral retinal specializations are as follows:

Immediately interior to the head capsule lies the pigment rim (PR), which is the outermost region of the retina. The PR does not contain ommatidia, but is a thick band of pigment cells, which acts to insulate the retina from extraneous light rays.

Next to the PR, up to four outermost ommatidial rows are devoid of the mechanosensory bristles. These comprise the bald ommatidia (BO).

The two outermost rows of these bald ommatidia in the dorsal half of the eye are specialized polarized light-detecting units – the dorsal rim ommatidia (DRO).

These specializations are generated in response to a gradient of Wg emanating from the surrounding HC, such that low level Wg signaling causes balding, intermediate levels of Wg signaling cause DRO formation and high levels of Wg signaling leads to PR formation (Tomlinson 2003). Clones of cells mutant for Wg signal transduction at the periphery of the eye are devoid of these specializations (Tomlinson 2003). Conversely, clones of cells ectopically expressing Wg at high levels caused scarring and balding of the surrounding ommatidia (Treisman and Rubin 1995, Tomlinson 2003). Sections through these eyes showed the presence of DRO like ommatidia in the clones which were generated in the dorsal half of the eye. These results suggest that Wg is required and sufficient to form all the peripheral retinal specializations (Tomlinson 2003).

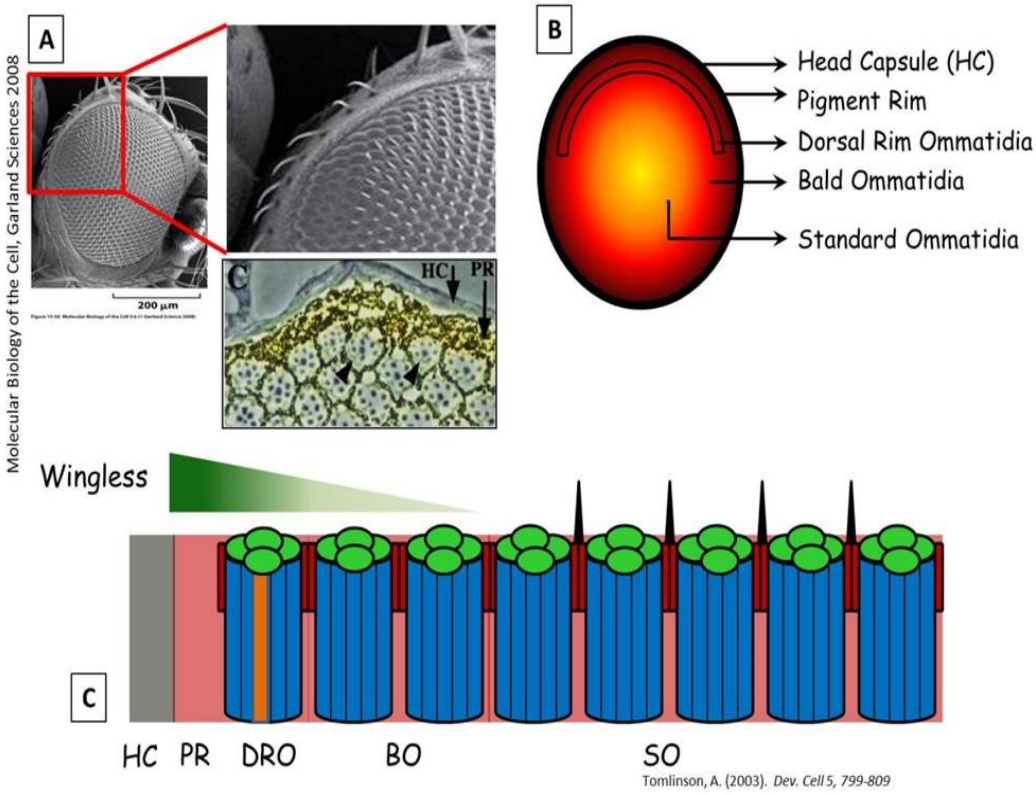

Figure 13: Peripheral specializations in the adult drosophila eye

A: The adult eye. Top inset panel highlights the bald ommatidia at the periphery. Lower inset shows the presence of a thick band of pigment cells – the pigment rim (PR) adjacent to the head capsule (HC). Image taken from (Tomlinson 2003)

B: Schematic representation of the peripheral specialization in the adult eye.

C: A gradient of Wg signaling leads to the peripheral patterning process.

The mechanism by which Wg signaling induces the formation of these morphological specializations is as follows:

Bald ommatidia (BO) – These ommatidia lack the characteristic mechanosensory bristle (Ready, Hanson et al. 1976). At the polar periphery, these bald ommatidia form the outermost ommatidial row, but at the anterior and posterior periphery, the bald region can extend as far as 5 rows of ommatidia into the main body of the eye (Tomlinson 2003). Bristle formation begins at the center of the eye and radiates outward. Bristles are composed of 4 cells – neuron, sheath shaft and socket, all believed to arise from a single mother cell, thus supporting clonal origin for the bristle cells as opposed to non-clonal accretion of the cells of the ommatidia. The bristle formation begins with the expression Achaete(Ac)/Scute(Sc) class of proneural transcription factors in the inter ommatidial cells (Campuzano and Modolell 1992). Once a single cell attains sufficient Ac/Sc expression levels, it represses the expression in the other cells of the cluster via N-mediated lateral inhibition and forms the bristle mother cell, also referred to as the Sensory Organ Precursor (SOP) cell (Artavanis-Tsakonas, Rand et al. 1999). For this SOP to form and develop as a neuron, Ac/Sc proteins are believed to heterodimerize with another helix-loop-helix transcription factor called Daughterless (Da); and this heterodimerization is essential for the subsequent neuronal differentiation (Cabrera and Alonso 1991, Campuzano and Modolell 1992). Low levels of Wg diffusing in from the HC at the periphery are sufficient to repress transcription of Da and Ac, thereby preventing the formation of the bristle neurons (Cadigan, Jou et al. 2002). The presence of predicted Pangolin (dTCF) binding sites in the Da 5'promoter region and intronic regions also supports the hypothesis that Wg directly represses Da transcription (Cadigan, Jou et al. 2002).

Dorsal Rim Ommatidia(DRO) – These are the two rows of ommatidia at the dorsal periphery of the eye, which are specialized to detect plane polarized light. These are reportedly utilized by insects to detect skylight polarization and thus adjust their flight orientation (Wernet, Labhart et al. 2003). The DRO have a number of distinguishing features – their central photoreceptors R7 and R8 both express the rhodopsin Rh3, as compared to Rh3/Rh4 in R7 and correspondingly Rh5/Rh6 in R8 cells. Another feature is the significantly enlarged rhabdomeres of both the central photoreceptors as compared to standard ommatidia. This presumably increases the sensitivity of polarized light detection. A third feature is the unusual projections of these ommatidia to the optic lobes (Fortini and Rubin 1991). Wg diffusing in from the HC, at intermediate levels transforms the outermost ommatidia to DRO in only the dorsal half of the eye. The spatial restriction of the DRO is because Wg acts in concert with the Iroquois complex (*Iro-C*) genes to lead to the expression of Hth, a homeodomain transcription factor in the central photoreceptors of the outermost ommatidia. As the Iro-C proteins are expressed solely in the dorsal half of the eye, only the dorsal periphery shows DRO formation (Wernet, Labhart et al. 2003). The Hth expression in the central photoreceptors R7/R8 is both required and sufficient to cause formation of the DRO. Misexpression of Hth in the main body ommatidia transforms them to DRO; and loss of Hth causes the DRO to become color-sensitive like the standard main body ommatidia (Wernet, Labhart et al. 2003).

Pigment Rim (PR) – The pigment rim is a thick band of pigment cells at the periphery of the retina, which acts to optically insulate the eye from extraneous light rays. Together with the basal pigment layer, the PR forms a niche which nestles the ommatidial array

(Tomlinson 2003). The PR formations is accomplished by removal of incomplete and degenerate optic units at the eye periphery, thus ensuring proper lattice construction throughout the retina (Ready, Hanson et al. 1976, Cadigan and Nusse 1996, Lin, Rogulja et al. 2004).

4 Mechanism of pigment rim formation

The PR is formed by apoptosis of perimeter ommatidia during pupal development, which causes the remaining 2°/3° pigment cells to coalesce and form the pigment rim. These ommatidia are frequently small and incomplete, lacking the appropriate axonal connections to the optic lobe (Ready, Hanson et al. 1976). Previous studies have indicated that high levels of Wg diffusing in from the head capsule led to this peripheral apoptosis (Lin, Rogulja et al. 2004). Blocking Wg signaling transduction at the periphery prevented this apoptosis and led to the survival of the peripheral ommatidia (Tomlinson 2003, Lin, Rogulja et al. 2004). Furthermore, it was shown that in response to the Wg signal, there is the expression of Snail class transcription factors along with Wg and Notum, in the cone cells and the surrounding 2°/3° pigment cells of the outermost ommatidia, which subsequently undergo apoptosis. This expression of the Snail class transcription factors in the peripheral ommatidia was reported to be required for the ommatidial apoptosis to occur (Lim and Tomlinson 2006).

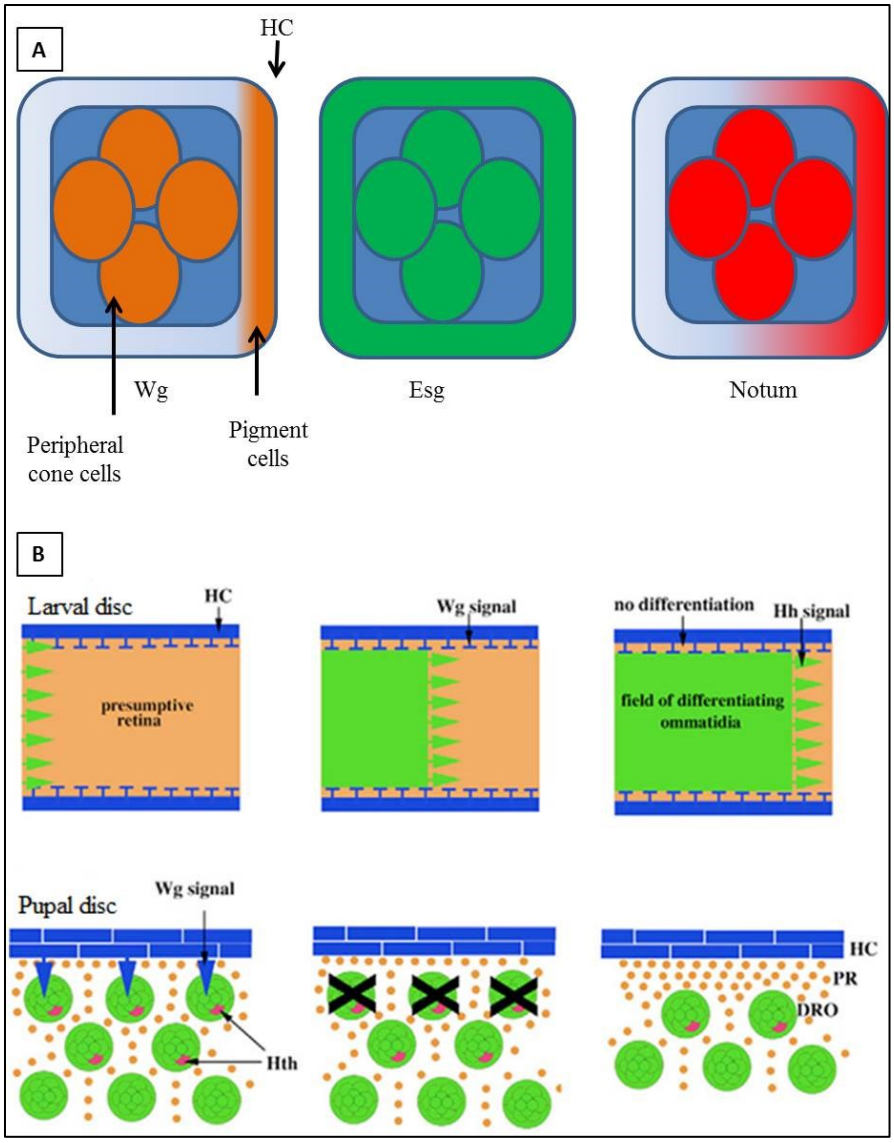

Figure 14: Schematic summary of the mechanism of pigment rim formation

A: Cartoon representation of the up-regulation of Wg, Esg and Notum, at 32hrsAPF in the cone cells of the outermost ommatidia. These proteins are also expressed in the surrounding 2°/3° pigment cell lattice to different extents as depicted.

B: Two sources of pigment cells contribute to the formation of the pigment rim: The cells that fail to undergo differentiation in the larval stages, at the very edges of the eye disc, and the 2°/3° pigment cells that surrounded the peripheral ommatidia that undergo developmental apoptosis. (Lim and Tomlinson 2006)

The Snail family of transcription factors is a group of DNA binding proteins with 4 to 6 conserved Zn finger domains. This family includes three proteins - Snail (Sna), Escargot (Esg) and Worniu (Wor) (Boulay, Dennefeld et al. 1987, Whiteley, Noguchi et al. 1992, Ashraf, Hu et al. 1999, Hemavathy, Ashraf et al. 2000). Sna is the prototypical member of the family, and was first isolated in a genetic screen for embryonic patterning mutations (Nusslein-Volhard and Wieschaus 1980). It is expressed in the ventral region of blastoderm stage embryos, and serves to repress neuroectodermal genes such as *single-minded* and *rhomboid* in the mesoderm (Boulay, Dennefeld et al. 1987, Jiang, Kosman et al. 1991, Leptin 1991). It has also been suggested to be involved in the regulation of genes involved in ventral cell invagination (Ip, Park et al. 1992, Hemavathy, Meng et al. 1997). Sna expression is functionally redundant with Esg and Wor in the development of neuroblasts and their asymmetric cell division (Ashraf, Hu et al. 1999, Ashraf and Ip 2001, Cai, Chia et al. 2001). Esg and Sna also exert redundant effects in wing cell fate determination, as *sna esg* double mutant embryos lack the wing marker gene *vg (*Fuse, Hirose et al. 1996). In some developmental aspects, these genes also show non redundant effects. Esg is expressed in the early imaginal discs as it is essential to maintain diploidy, unlike the polytene larval epidermal cells (Hayashi, Hirose et al. 1993). It regulates tracheal branch fusion by regulation the expression and accumulation of DE-cadherin at

the tips of the tracheal branches (Tanaka-Matakatsu, Uemura et al. 1996). Esg is also reported to be involved in the tightening of the 2°/3° pigment cell lattice in later pupal development stages of the eye (Lim and Tomlinson 2006). Mutations in Wor cause a failure in the shortening of the larval brainstem, thus affecting brain development (Ashraf, Ganguly et al. 2004).

Previous studies showed that in response to the Wg diffusing in from the HC, Wg expression is up regulated in the outermost ommatidia. These ommatidia subsequently undergo apoptotic removal (Lin, Rogulja et al. 2004). Later experiments showed that this secondary Wg expression occurs at a very specific developmental time point in a subset of the cells of the outermost ommatidia, namely the cone cells and the 2°/3° pigment cells. Concomitantly, the proteins of the Snail family of transcription factors (Esg, Sna and Wor) were also shown to be expressed in these peripheral cone cells. This expression pattern was shown to be dependent on Wg signaling, and is required for the peripheral apoptosis (Lim and Tomlinson 2006). The focus of my thesis is to understand how this set of gene expressions in a subset of the cells of the ommatidia, leads to the apoptosis of the entire ommatidium.

Artavanis-Tsakonas, S., M. D. Rand and R. J. Lake (1999). "Notch signaling: cell fate control and signal integration in development." Science **284**(5415): 770-776.

Ashburner, M. and J. Roote (2007). "Culture of Drosophila: The Laboratory Setup." Cold Spring Harbor Protocols **2007**(3): pdb.ip34.

Ashe, H. L. and J. Briscoe (2006). "The interpretation of morphogen gradients." Development **133**(3): 385-394.

Ashraf, S. I., A. Ganguly, J. Roote and Y. T. Ip (2004). "Worniu, a Snail family zinc-finger protein, is required for brain development in Drosophila." Dev Dyn **231**(2): 379-386.

Ashraf, S. I., X. Hu, J. Roote and Y. T. Ip (1999). "The mesoderm determinant snail collaborates with related zinc-finger proteins to control Drosophila neurogenesis." EMBO J **18**(22): 6426-6438.

Ashraf, S. I. and Y. T. Ip (2001). "The Snail protein family regulates neuroblast expression of inscuteable and string, genes involved in asymmetry and cell division in Drosophila." Development **128**(23): 4757-4767.

Baig-Lewis, S., W. Peterson-Nedry and M. Wehrli (2007). "Wingless/Wnt signal transduction requires distinct initiation and amplification steps that both depend on Arrow/LRP." Dev Biol **306**(1): 94-111.

Baker, N. E. (1988). "Localization of transcripts from the wingless gene in whole Drosophila embryos." Development **103**(2): 289-298.

Baker, N. E. and L. C. Firth (2011). "Retinal determination genes function along with cell-cell signals to regulate Drosophila eye development: examples of multi-layered regulation by master regulators." Bioessays **33**(7): 538-546.

Baker, N. E., M. Mlodzik and G. M. Rubin (1990). "Spacing differentiation in the developing Drosophila eye: a fibrinogen-related lateral inhibitor encoded by scabrous." Science **250**(4986): 1370-1377.

Baker, N. E., S. Yu and D. Han (1996). "Evolution of proneural atonal expression during distinct regulatory phases in the developing Drosophila eye." Curr Biol **6**(10): 1290-1301.

Baker, N. E. and S. Y. Yu (1997). "Proneural function of neurogenic genes in the developing Drosophila eye." Curr Biol **7**(2): 122-132.

Bao, S. and R. Cagan (2005). "Preferential adhesion mediated by Hibris and Roughest regulates morphogenesis and patterning in the Drosophila eye." Dev Cell **8**(6): 925-935.

Baonza, A. and M. Freeman (2001). "Notch signalling and the initiation of neural development in the Drosophila eye." Development **128**(20): 3889-3898.

Baonza, A. and M. Freeman (2002). "Control of Drosophila eye specification by Wingless signalling." Development **129**(23): 5313-5322.

Basler, K., B. Christen and E. Hafen (1991). "Ligand-independent activation of the sevenless receptor tyrosine kinase changes the fate of cells in the developing Drosophila eye." Cell **64**(6): 1069-1081.

Behrens, J., B. A. Jerchow, M. Wurtele, J. Grimm, C. Asbrand, R. Wirtz, M. Kuhl, D. Wedlich and W. Birchmeier (1998). "Functional interaction of an axin homolog, conductin, with beta-catenin, APC, and GSK3beta." Science **280**(5363): 596-599.

Bejsovec, A. (2013). "Wingless/Wnt signaling in Drosophila: The pattern and the pathway." Mol Reprod Dev.

Benchabane, H., E. G. Hughes, C. M. Takacs, J. R. Baird and Y. Ahmed (2008). "Adenomatous polyposis coli is present near the minimal level required for accurate graded responses to the Wingless morphogen." Development **135**(5): 963-971.

Bessa, J. and F. Casares (2005). "Restricted teashirt expression confers eye-specific responsiveness to Dpp and Wg signals during eye specification in Drosophila." Development **132**(22): 5011-5020.

Bessa, J., B. Gebelein, F. Pichaud, F. Casares and R. S. Mann (2002). "Combinatorial control of Drosophila eye development by eyeless, homothorax, and teashirt." Genes Dev **16**(18): 2415-2427.

Bhanot, P., M. Brink, C. H. Samos, J. C. Hsieh, Y. Wang, J. P. Macke, D. Andrew, J. Nathans and R. Nusse (1996). "A new member of the frizzled family from Drosophila functions as a Wingless receptor." Nature **382**(6588): 225-230.

Bhanot, P., M. Fish, J. A. Jemison, R. Nusse, J. Nathans and K. M. Cadigan (1999). "Frizzled and Dfrizzled-2 function as redundant receptors for Wingless during Drosophila embryonic development." Development **126**(18): 4175-4186.

Blackman, R. K., M. Sanicola, L. A. Raftery, T. Gillevet and W. M. Gelbart (1991). "An extensive 3' cis-regulatory region directs the imaginal disk expression of decapentaplegic, a member of the TGF-beta family in Drosophila." Development **111**(3): 657-666.

Bodmer, R., S. Barbel, S. Sheperd, J. W. Jack, L. Y. Jan and Y. N. Jan (1987). "Transformation of sensory organs by mutations of the cut locus of D. melanogaster." Cell **51**(2): 293-307.

Borod, E. R. and U. Heberlein (1998). "Mutual regulation of decapentaplegic and hedgehog during the initiation of differentiation in the Drosophila retina." Dev Biol **197**(2): 187-197.

Boulay, J. L., C. Dennefeld and A. Alberga (1987). "The Drosophila developmental gene snail encodes a protein with nucleic acid binding fingers." Nature **330**(6146): 395-398.

Bourouis, M., P. Moore, L. Ruel, Y. Grau, P. Heitzler and P. Simpson (1990). "An early embryonic product of the gene shaggy encodes a serine/threonine protein kinase related to the CDC28/cdc2+ subfamily." EMBO J **9**(9): 2877-2884.

Brand, A. H. and N. Perrimon (1993). "Targeted gene expression as a means of altering cell fates and generating dominant phenotypes." Development **118**(2): 401-415.

Brembeck, F. H., T. Schwarz-Romond, J. Bakkers, S. Wilhelm, M. Hammerschmidt and W. Birchmeier (2004). "Essential role of BCL9-2 in the switch between beta-catenin's adhesive and transcriptional functions." Genes Dev **18**(18): 2225-2230.

Brown, N. L., C. A. Sattler, S. W. Paddock and S. B. Carroll (1995). "Hairy and emc negatively regulate morphogenetic furrow progression in the Drosophila eye." Cell **80**(6): 879-887.

Bruckner, K., L. Perez, H. Clausen and S. Cohen (2000). "Glycosyltransferase activity of Fringe modulates Notch-Delta interactions." Nature **406**(6794): 411-415.

Bui, Q. T., J. E. Zimmerman, H. Liu, G. L. Gray-Board and N. M. Bonini (2000). "Functional analysis of an eye enhancer of the Drosophila eyes absent gene: differential regulation by eye specification genes." Dev Biol **221**(2): 355-364.

Burke, R. and K. Basler (1996). "Hedgehog-dependent patterning in the Drosophila eye can occur in the absence of Dpp signaling." Dev Biol **179**(2): 360-368.

Cabrera, C. V. and M. C. Alonso (1991). "Transcriptional activation by heterodimers of the achaete-scute and daughterless gene products of Drosophila." EMBO J **10**(10): 2965-2973.

Cadigan, K. M., A. D. Jou and R. Nusse (2002). "Wingless blocks bristle formation and morphogenetic furrow progression in the eye through repression of Daughterless." Development **129**(14): 3393-3402.

Cadigan, K. M. and R. Nusse (1996). "wingless signaling in the Drosophila eye and embryonic epidermis." Development **122**(9): 2801-2812.

Cagan, R. L. and D. F. Ready (1989). "The emergence of order in the Drosophila pupal retina." Dev Biol **136**(2): 346-362.

Cagan, R. L. and D. F. Ready (1989). "Notch is required for successive cell decisions in the developing Drosophila retina." Genes Dev **3**(8): 1099-1112.

Cai, Y., W. Chia and X. Yang (2001). "A family of snail-related zinc finger proteins regulates two distinct and parallel mechanisms that mediate Drosophila neuroblast asymmetric divisions." EMBO J **20**(7): 1704-1714.

Campuzano, S. and J. Modolell (1992). "Patterning of the Drosophila nervous system: the achaete-scute gene complex." Trends Genet **8**(6): 202-208.

Cavallo, R. A., R. T. Cox, M. M. Moline, J. Roose, G. A. Polevoy, H. Clevers, M. Peifer and A. Bejsovec (1998). "Drosophila Tcf and Groucho interact to repress Wingless signalling activity." Nature **395**(6702): 604-608.

Cavodeassi, F., R. Diez Del Corral, S. Campuzano and M. Dominguez (1999). "Compartments and organising boundaries in the Drosophila eye: the role of the homeodomain Iroquois proteins." Development **126**(22): 4933-4942.

Chang, H. C., N. M. Solomon, D. A. Wassarman, F. D. Karim, M. Therrien, G. M. Rubin and T. Wolff (1995). "phyllopod functions in the fate determination of a subset of photoreceptors in Drosophila." Cell **80**(3): 463-472.

Chanut, F. and U. Heberlein (1995). "Role of the morphogenetic furrow in establishing polarity in the Drosophila eye." Development **121**(12): 4085-4094.

Chanut, F., A. Luk and U. Heberlein (2000). "A screen for dominant modifiers of ro(Dom), a mutation that disrupts morphogenetic furrow progression in Drosophila, identifies groucho and hairless as regulators of atonal expression." Genetics **156**(3): 1203-1217.

Chao, J. L., Y. C. Tsai, S. J. Chiu and Y. H. Sun (2004). "Localized Notch signal acts through eyg and upd to promote global growth in Drosophila eye." Development **131**(16): 3839-3847.

Chen, R., G. Halder, Z. Zhang and G. Mardon (1999). "Signaling by the TGF-beta homolog decapentaplegic functions reiteratively within the network of genes controlling retinal cell fate determination in Drosophila." Development **126**(5): 935-943.

Cho, K. O., J. Chern, S. Izaddoost and K. W. Choi (2000). "Novel signaling from the peripodial membrane is essential for eye disc patterning in Drosophila." Cell **103**(2): 331-342.

Cho, K. O. and K. W. Choi (1998). "Fringe is essential for mirror symmetry and morphogenesis in the Drosophila eye." Nature **396**(6708): 272-276.

Cliffe, A., F. Hamada and M. Bienz (2003). "A role of Dishevelled in relocating Axin to the plasma membrane during wingless signaling." Curr Biol **13**(11): 960-966.

Clos, J., J. T. Westwood, P. B. Becker, S. Wilson, K. Lambert and C. Wu (1990). "Molecular cloning and expression of a hexameric Drosophila heat shock factor subject to negative regulation." Cell **63**(5): 1085-1097.

Cohen, M., J. Briscoe and R. Blassberg (2013). "Morphogen interpretation: the transcriptional logic of neural tube patterning." Curr Opin Genet Dev **23**(4): 423-428.

Cooper, M. T. and S. J. Bray (2000). "R7 photoreceptor specification requires Notch activity." Curr Biol **10**(23): 1507-1510.

Couso, J. P., S. A. Bishop and A. Martinez Arias (1994). "The wingless signalling pathway and the patterning of the wing margin in Drosophila." Development **120**(3): 621-636.

Cox, R. T., C. Kirkpatrick and M. Peifer (1996). "Armadillo is required for adherens junction assembly, cell polarity, and morphogenesis during Drosophila embryogenesis." J Cell Biol **134**(1): 133-148.

Crick, F. (1970). "Diffusion in embryogenesis." Nature **225**(5231): 420-422.

Curtiss, J. and M. Mlodzik (2000). "Morphogenetic furrow initiation and progression during eye development in Drosophila: the roles of decapentaplegic, hedgehog and eyes absent." Development **127**(6): 1325-1336.

Czerny, T., G. Halder, U. Kloter, A. Souabni, W. J. Gehring and M. Busslinger (1999). "twin of eyeless, a second Pax-6 gene of Drosophila, acts upstream of eyeless in the control of eye development." Mol Cell **3**(3): 297-307.

Dajani, R., E. Fraser, S. M. Roe, M. Yeo, V. M. Good, V. Thompson, T. C. Dale and L. H. Pearl (2003). "Structural basis for recruitment of glycogen synthase kinase 3beta to the axin-APC scaffold complex." EMBO J **22**(3): 494-501.

Das, G., J. Reynolds-Kenneally and M. Mlodzik (2002). "The atypical cadherin Flamingo links Frizzled and Notch signaling in planar polarity establishment in the Drosophila eye." Dev Cell **2**(5): 655-666.

DasGupta, R., A. Kaykas, R. T. Moon and N. Perrimon (2005). "Functional genomic analysis of the Wnt-wingless signaling pathway." Science **308**(5723): 826-833.

de Celis, J. F., A. Garcia-Bellido and S. J. Bray (1996). "Activation and function of Notch at the dorsal-ventral boundary of the wing imaginal disc." Development **122**(1): 359-369.

Desplan, C. (1997). "Eye development: governed by a dictator or a junta?" Cell **91**(7): 861-864.

Diaz-Benjumea, F. J. and S. M. Cohen (1995). "Serrate signals through Notch to establish a Wingless-dependent organizer at the dorsal/ventral compartment boundary of the Drosophila wing." Development **121**(12): 4215-4225.

Dickson, B. J., M. Dominguez, A. van der Straten and E. Hafen (1995). "Control of Drosophila photoreceptor cell fates by phyllopod, a novel nuclear protein acting downstream of the Raf kinase." Cell **80**(3): 453-462.

Dokucu, M. E., S. L. Zipursky and R. L. Cagan (1996). "Atonal, rough and the resolution of proneural clusters in the developing Drosophila retina." Development **122**(12): 4139-4147.

Dominguez, M. and F. Casares (2005). "Organ specification-growth control connection: new in-sights from the Drosophila eye-antennal disc." Dev Dyn **232**(3): 673-684.

Dominguez, M. and J. F. de Celis (1998). "A dorsal/ventral boundary established by Notch controls growth and polarity in the Drosophila eye." Nature **396**(6708): 276-278.

Dominguez, M., D. Ferres-Marco, F. J. Gutierrez-Avino, S. A. Speicher and M. Beneyto (2004). "Growth and specification of the eye are controlled independently by Eyegone and Eyeless in Drosophila melanogaster." Nat Genet **36**(1): 31-39.

Dominguez, M. and E. Hafen (1997). "Hedgehog directly controls initiation and propagation of retinal differentiation in the Drosophila eye." Genes Dev **11**(23): 3254-3264.

Dominguez, M., J. D. Wasserman and M. Freeman (1998). "Multiple functions of the EGF receptor in Drosophila eye development." Curr Biol **8**(19): 1039-1048.

Dong, P. D., J. Chu and G. Panganiban (2000). "Coexpression of the homeobox genes Distal-less and homothorax determines Drosophila antennal identity." Development **127**(2): 209-216.

Dougan, S. T., R. M. Warga, D. A. Kane, A. F. Schier and W. S. Talbot (2003). "The role of the zebrafish nodal-related genes squint and cyclops in patterning of mesendoderm." Development **130**(9): 1837-1851.

Entchev, E. V., A. Schwabedissen and M. Gonzalez-Gaitan (2000). "Gradient formation of the TGF-beta homolog Dpp." Cell **103**(6): 981-991.

Feldman, B., M. A. Gates, E. S. Egan, S. T. Dougan, G. Rennebeck, H. I. Sirotkin, A. F. Schier and W. S. Talbot (1998). "Zebrafish organizer development and germ-layer formation require nodal-related signals." Nature **395**(6698): 181-185.

Fiedler, M., C. Mendoza-Topaz, T. J. Rutherford, J. Mieszczanek and M. Bienz (2011). "Dishevelled interacts with the DIX domain polymerization interface of Axin to interfere with its function in down-regulating beta-catenin." Proc Natl Acad Sci U S A **108**(5): 1937-1942.

Firth, L. C., A. Bhattacharya and N. E. Baker (2010). "Cell cycle arrest by a gradient of Dpp signaling during Drosophila eye development." BMC Dev Biol **10**: 28.

Flores, G. V., A. Daga, H. R. Kalhor and U. Banerjee (1998). "Lozenge is expressed in pluripotent precursor cells and patterns multiple cell types in the Drosophila eye through the control of cell-specific transcription factors." Development **125**(18): 3681-3687.

Flores, G. V., H. Duan, H. Yan, R. Nagaraj, W. Fu, Y. Zou, M. Noll and U. Banerjee (2000). "Combinatorial signaling in the specification of unique cell fates." Cell **103**(1): 75-85.

Fortini, M. E., I. Rebay, L. A. Caron and S. Artavanis-Tsakonas (1993). "An activated Notch receptor blocks cell-fate commitment in the developing Drosophila eye." Nature **365**(6446): 555-557.

Fortini, M. E. and G. M. Rubin (1991). "The optic lobe projection pattern of polarization-sensitive photoreceptor cells in Drosophila melanogaster." Cell Tissue Res **265**(1): 185-191.

Fradkin, L. G., J. N. Noordermeer and R. Nusse (1995). "The Drosophila Wnt protein DWnt-3 is a secreted glycoprotein localized on the axon tracts of the embryonic CNS." Dev Biol **168**(1): 202-213.

Fradkin, L. G., M. van Schie, R. R. Wouda, A. de Jong, J. T. Kamphorst, M. Radjkoemar-Bansraj and J. N. Noordermeer (2004). "The Drosophila Wnt5 protein mediates selective axon fasciculation in the embryonic central nervous system." Dev Biol **272**(2): 362-375.

Freeman, M. (1996). "Reiterative use of the EGF receptor triggers differentiation of all cell types in the Drosophila eye." Cell **87**(4): 651-660.

Fuse, N., S. Hirose and S. Hayashi (1996). "Determination of wing cell fate by the escargot and snail genes in Drosophila." Development **122**(4): 1059-1067.

Garcia-Bellido, A., P. Ripoll and G. Morata (1976). "Developmental compartmentalization in the dorsal mesothoracic disc of Drosophila." Dev Biol **48**(1): 132-147.

Gottardi, C. J. and M. Peifer (2008). "Terminal regions of beta-catenin come into view." Structure **16**(3): 336-338.

Gradilla, A. C. and I. Guerrero (2013). "Cytoneme-mediated cell-to-cell signaling during development." Cell Tissue Res **352**(1): 59-66.

Greco, V., M. Hannus and S. Eaton (2001). "Argosomes: a potential vehicle for the spread of morphogens through epithelia." Cell **106**(5): 633-645.

Green, P., A. Y. Hartenstein and V. Hartenstein (1993). "The embryonic development of the Drosophila visual system." Cell Tissue Res **273**(3): 583-598.

Greenwood, S. and G. Struhl (1999). "Progression of the morphogenetic furrow in the Drosophila eye: the roles of Hedgehog, Decapentaplegic and the Raf pathway." Development **126**(24): 5795-5808.

Grzeschik, N. A. and E. Knust (2005). "IrreC/rst-mediated cell sorting during Drosophila pupal eye development depends on proper localisation of DE-cadherin." Development **132**(9): 2035-2045.

Gurdon, J. B. and P. Y. Bourillot (2001). "Morphogen gradient interpretation." Nature **413**(6858): 797-803.

Hafen, E., K. Basler, J. E. Edstroem and G. M. Rubin (1987). "Sevenless, a cell-specific homeotic gene of Drosophila, encodes a putative transmembrane receptor with a tyrosine kinase domain." Science **236**(4797): 55-63.

Hamada, F., Y. Tomoyasu, Y. Takatsu, M. Nakamura, S. Nagai, A. Suzuki, F. Fujita, H. Shibuya, K. Toyoshima, N. Ueno and T. Akiyama (1999). "Negative regulation of Wingless signaling by D-axin, a Drosophila homolog of axin." Science **283**(5408): 1739-1742.

Hart, M. J., R. de los Santos, I. N. Albert, B. Rubinfeld and P. Polakis (1998). "Downregulation of beta-catenin by human Axin and its association with the APC tumor suppressor, beta-catenin and GSK3 beta." Curr Biol **8**(10): 573-581.

Hayashi, S., S. Hirose, T. Metcalfe and A. D. Shirras (1993). "Control of imaginal cell development by the escargot gene of Drosophila." Development **118**(1): 105-115.

Hayashi, T. and K. Saigo (2001). "Diversification of cell types in the Drosophila eye by differential expression of prepattern genes." Mech Dev **108**(1-2): 13-27.

Haynie, J. L. and P. J. Bryant (1986). "Development of the eye-antenna imaginal disc and morphogenesis of the adult head in Drosophila melanogaster." J Exp Zool **237**(3): 293-308.

Hazelett, D. J., M. Bourouis, U. Walldorf and J. E. Treisman (1998). "decapentaplegic and wingless are regulated by eyes absent and eyegone and interact to direct the pattern of retinal differentiation in the eye disc." Development **125**(18): 3741-3751.

He, X., M. Semenov, K. Tamai and X. Zeng (2004). "LDL receptor-related proteins 5 and 6 in Wnt/beta-catenin signaling: arrows point the way." Development **131**(8): 1663-1677.

Heberlein, U., T. Wolff and G. M. Rubin (1993). "The TGF beta homolog dpp and the segment polarity gene hedgehog are required for propagation of a morphogenetic wave in the Drosophila retina." Cell **75**(5): 913-926.

Hemavathy, K., S. I. Ashraf and Y. T. Ip (2000). "Snail/slug family of repressors: slowly going into the fast lane of development and cancer." Gene **257**(1): 1-12.

Hemavathy, K., X. Meng and Y. T. Ip (1997). "Differential regulation of gastrulation and neuroectodermal gene expression by Snail in the Drosophila embryo." Development **124**(19): 3683-3691.

Henderson, B. R. (2000). "Nuclear-cytoplasmic shuttling of APC regulates beta-catenin subcellular localization and turnover." Nat Cell Biol **2**(9): 653-660.

Horsfield, J., A. Penton, J. Secombe, F. M. Hoffman and H. Richardson (1998). "decapentaplegic is required for arrest in G1 phase during Drosophila eye development." Development **125**(24): 5069-5078.

Hsiung, F. and K. Moses (2002). "Retinal development in Drosophila: specifying the first neuron." Hum Mol Genet **11**(10): 1207-1214.

Hsiung, F., F. A. Ramirez-Weber, D. D. Iwaki and T. B. Kornberg (2005). "Dependence of Drosophila wing imaginal disc cytonemes on Decapentaplegic." Nature **437**(7058): 560-563.

Huber, A. H., W. J. Nelson and W. I. Weis (1997). "Three-dimensional structure of the armadillo repeat region of beta-catenin." Cell **90**(5): 871-882.

Ikeda, S., S. Kishida, H. Yamamoto, H. Murai, S. Koyama and A. Kikuchi (1998). "Axin, a negative regulator of the Wnt signaling pathway, forms a complex with GSK-3beta and beta-catenin and promotes GSK-3beta-dependent phosphorylation of beta-catenin." EMBO J **17**(5): 1371-1384.

Ip, Y. T., R. E. Park, D. Kosman, K. Yazdanbakhsh and M. Levine (1992). "dorsal-twist interactions establish snail expression in the presumptive mesoderm of the Drosophila embryo." Genes Dev **6**(8): 1518-1530.

Jarman, A. P., E. H. Grell, L. Ackerman, L. Y. Jan and Y. N. Jan (1994). "Atonal is the proneural gene for Drosophila photoreceptors." Nature **369**(6479): 398-400.

Jarman, A. P., Y. Sun, L. Y. Jan and Y. N. Jan (1995). "Role of the proneural gene, atonal, in formation of Drosophila chordotonal organs and photoreceptors." Development **121**(7): 2019-2030.

Jho, E., S. Lomvardas and F. Costantini (1999). "A GSK3beta phosphorylation site in axin modulates interaction with beta-catenin and Tcf-mediated gene expression." Biochem Biophys Res Commun **266**(1): 28-35.

Jiang, J., D. Kosman, Y. T. Ip and M. Levine (1991). "The dorsal morphogen gradient regulates the mesoderm determinant twist in early Drosophila embryos." Genes Dev **5**(10): 1881-1891.

Kammermeier, L., R. Leemans, F. Hirth, S. Flister, U. Wenger, U. Walldorf, W. J. Gehring and H. Reichert (2001). "Differential expression and function of the Drosophila Pax6 genes eyeless and twin of eyeless in embryonic central nervous system development." Mech Dev **103**(1-2): 71-78.

Kawahara, K., T. Morishita, T. Nakamura, F. Hamada, K. Toyoshima and T. Akiyama (2000). "Down-regulation of beta-catenin by the colorectal tumor suppressor APC requires association with Axin and beta-catenin." J Biol Chem **275**(12): 8369-8374.

Kenyon, K. L., S. S. Ranade, J. Curtiss, M. Mlodzik and F. Pignoni (2003). "Coordinating proliferation and tissue specification to promote regional identity in the Drosophila head." Dev Cell **5**(3): 403-414.

Kicheva, A., P. Pantazis, T. Bollenbach, Y. Kalaidzidis, T. Bittig, F. Julicher and M. Gonzalez-Gaitan (2007). "Kinetics of morphogen gradient formation." Science **315**(5811): 521-525.

Kikuchi, A. and S. Kishida (2006). "[Abnormal regulation of beta-catenin degradation through ubiquitination as a mechanism of carcinogenesis]." Tanpakushitsu Kakusan Koso **51**(10 Suppl): 1271-1276.

Kim, J., A. Sebring, J. J. Esch, M. E. Kraus, K. Vorwerk, J. Magee and S. B. Carroll (1996). "Integration of positional signals and regulation of wing formation and identity by Drosophila vestigial gene." Nature **382**(6587): 133-138.

Kim, S. E., H. Huang, M. Zhao, X. Zhang, A. Zhang, M. V. Semonov, B. T. MacDonald, X. Zhang, J. Garcia Abreu, L. Peng and X. He (2013). "Wnt stabilization of beta-catenin reveals principles for morphogen receptor-scaffold assemblies." Science **340**(6134): 867-870.

Kimelman, D. and W. Xu (2006). "beta-catenin destruction complex: insights and questions from a structural perspective." Oncogene **25**(57): 7482-7491.

Kishida, S., H. Yamamoto, S. Hino, S. Ikeda, M. Kishida and A. Kikuchi (1999). "DIX domains of Dvl and axin are necessary for protein interactions and their ability to regulate beta-catenin stability." Mol Cell Biol **19**(6): 4414-4422.

Kooh, P. J., R. G. Fehon and M. A. Muskavitch (1993). "Implications of dynamic patterns of Delta and Notch expression for cellular interactions during Drosophila development." Development **117**(2): 493-507.

Kozopas, K. M., C. H. Samos and R. Nusse (1998). "DWnt-2, a Drosophila Wnt gene required for the development of the male reproductive tract, specifies a sexually dimorphic cell fate." Genes Dev **12**(8): 1155-1165.

Kuhl, M., L. C. Sheldahl, C. C. Malbon and R. T. Moon (2000). "Ca(2+)/calmodulin-dependent protein kinase II is stimulated by Wnt and Frizzled homologs and promotes ventral cell fates in Xenopus." J Biol Chem **275**(17): 12701-12711.

Kuhl, M., L. C. Sheldahl, M. Park, J. R. Miller and R. T. Moon (2000). "The Wnt/Ca2+ pathway: a new vertebrate Wnt signaling pathway takes shape." Trends Genet **16**(7): 279-283.

Kumar, J. P. and K. Moses (2001). "Expression of evolutionarily conserved eye specification genes during Drosophila embryogenesis." Dev Genes Evol **211**(8-9): 406-414.

Kumar, J. P., M. Tio, F. Hsiung, S. Akopyan, L. Gabay, R. Seger, B. Z. Shilo and K. Moses (1998). "Dissecting the roles of the Drosophila EGF receptor in eye development and MAP kinase activation." Development **125**(19): 3875-3885.

Lander, A. D., Q. Nie and F. Y. Wan (2007). "Membrane-associated non-receptors and morphogen gradients." Bull Math Biol **69**(1): 33-54.

Lee, E., A. Salic, R. Kruger, R. Heinrich and M. W. Kirschner (2003). "The roles of APC and Axin derived from experimental and theoretical analysis of the Wnt pathway." PLoS Biol **1**(1): E10.

Lei, J. and Y. Song (2010). "Mathematical model of the formation of morphogen gradients through membrane-associated non-receptors." Bull Math Biol **72**(4): 805-829.

Leptin, M. (1991). "twist and snail as positive and negative regulators during Drosophila mesoderm development." Genes Dev **5**(9): 1568-1576.

Li, S., Y. Li, R. W. Carthew and Z. C. Lai (1997). "Photoreceptor cell differentiation requires regulated proteolysis of the transcriptional repressor Tramtrack." Cell **90**(3): 469-478.

Li, S., C. Xu and R. W. Carthew (2002). "Phyllopod acts as an adaptor protein to link the sina ubiquitin ligase to the substrate protein tramtrack." Mol Cell Biol **22**(19): 6854-6865.

Li, Y. and N. E. Baker (2001). "Proneural enhancement by Notch overcomes Suppressor-of-Hairless repressor function in the developing Drosophila eye." Curr Biol **11**(5): 330-338.

Lim, H. Y. and A. Tomlinson (2006). "Organization of the peripheral fly eye: the roles of Snail family transcription factors in peripheral retinal apoptosis." Development **133**(18): 3529-3537.

Lin, H. V., A. Rogulja and K. M. Cadigan (2004). "Wingless eliminates ommatidia from the edge of the developing eye through activation of apoptosis." Development **131**(10): 2409-2418.

Llimargas, M. and P. A. Lawrence (2001). "Seven Wnt homologues in Drosophila: a case study of the developing tracheae." Proc Natl Acad Sci U S A **98**(25): 14487-14492.

Logan, C. Y. and R. Nusse (2004). "The Wnt signaling pathway in development and disease." Annu Rev Cell Dev Biol **20**: 781-810.

Lu, W., C. Lin, M. J. Roberts, W. R. Waud, G. A. Piazza and Y. Li (2011). "Niclosamide suppresses cancer cell growth by inducing Wnt co-receptor LRP6 degradation and inhibiting the Wnt/beta-catenin pathway." PLoS One **6**(12): e29290.

Luo, W., A. Peterson, B. A. Garcia, G. Coombs, B. Kofahl, R. Heinrich, J. Shabanowitz, D. F. Hunt, H. J. Yost and D. M. Virshup (2007). "Protein phosphatase 1 regulates assembly and function of the beta-catenin degradation complex." EMBO J **26**(6): 1511-1521.

Ma, C. and K. Moses (1995). "Wingless and patched are negative regulators of the morphogenetic furrow and can affect tissue polarity in the developing Drosophila compound eye." Development **121**(8): 2279-2289.

Mao, J., J. Wang, B. Liu, W. Pan, G. H. Farr, 3rd, C. Flynn, H. Yuan, S. Takada, D. Kimelman, L. Li and D. Wu (2001). "Low-density lipoprotein receptor-related protein-5 binds to Axin and regulates the canonical Wnt signaling pathway." Mol Cell **7**(4): 801-809.

Masucci, J. D., R. J. Miltenberger and F. M. Hoffmann (1990). "Pattern-specific expression of the Drosophila decapentaplegic gene in imaginal disks is regulated by 3' cis-regulatory elements." Genes Dev **4**(11): 2011-2023.

Maurel-Zaffran, C. and J. E. Treisman (2000). "pannier acts upstream of wingless to direct dorsal eye disc development in Drosophila." Development **127**(5): 1007-1016.

Mavromatakis, Y. E. and A. Tomlinson (2013). "Switching cell fates in the developing Drosophila eye." Development **140**(21): 4353-4361.

McCrea, P. D., C. W. Turck and B. Gumbiner (1991). "A homolog of the armadillo protein in Drosophila (plakoglobin) associated with E-cadherin." Science **254**(5036): 1359-1361.

Mendoza-Topaz, C., J. Mieszczanek and M. Bienz (2011). "The Adenomatous polyposis coli tumour suppressor is essential for Axin complex assembly and function and opposes Axin's interaction with Dishevelled." Open Biol **1**(3): 110013.

Metcalfe, C. and M. Bienz (2011). "Inhibition of GSK3 by Wnt signalling--two contrasting models." J Cell Sci **124**(Pt 21): 3537-3544.

Michaut, L., S. Flister, M. Neeb, K. P. White, U. Certa and W. J. Gehring (2003). "Analysis of the eye developmental pathway in Drosophila using DNA microarrays." Proc Natl Acad Sci U S A **100**(7): 4024-4029.

Miller, A. C., E. L. Lyons and T. G. Herman (2009). "cis-Inhibition of Notch by endogenous Delta biases the outcome of lateral inhibition." Curr Biol **19**(16): 1378-1383.

Miller, D. T. and R. L. Cagan (1998). "Local induction of patterning and programmed cell death in the developing Drosophila retina." Development **125**(12): 2327-2335.

Mitchell, B., J. L. Stubbs, F. Huisman, P. Taborek, C. Yu and C. Kintner (2009). "The PCP pathway instructs the planar orientation of ciliated cells in the Xenopus larval skin." Curr Biol **19**(11): 924-929.

Morata, G. and P. A. Lawrence (1979). "Development of the eye-antenna imaginal disc of Drosophila." Dev Biol **70**(2): 355-371.

Muller, P., K. W. Rogers, S. R. Yu, M. Brand and A. F. Schier (2013). "Morphogen transport." Development **140**(8): 1621-1638.

Nakamura, T., F. Hamada, T. Ishidate, K. Anai, K. Kawahara, K. Toyoshima and T. Akiyama (1998). "Axin, an inhibitor of the Wnt signalling pathway, interacts with beta-catenin, GSK-3beta and APC and reduces the beta-catenin level." Genes Cells **3**(6): 395-403.

Neckameyer, W. S. and K. J. Argue (2013). "Comparative approaches to the study of physiology: Drosophila as a physiological tool." Am J Physiol Regul Integr Comp Physiol **304**(3): R177-188.

Nellen, D., R. Burke, G. Struhl and K. Basler (1996). "Direct and long-range action of a DPP morphogen gradient." Cell **85**(3): 357-368.

Neumann, C. J. and S. M. Cohen (1997). "Long-range action of Wingless organizes the dorsal-ventral axis of the Drosophila wing." Development **124**(4): 871-880.

Niwa, N., Y. Hiromi and M. Okabe (2004). "A conserved developmental program for sensory organ formation in Drosophila melanogaster." Nat Genet **36**(3): 293-297.

Nusse, R. and H. E. Varmus (1982). "Many tumors induced by the mouse mammary tumor virus contain a provirus integrated in the same region of the host genome." Cell **31**(1): 99-109.

Nusslein-Volhard, C. and E. Wieschaus (1980). "Mutations affecting segment number and polarity in Drosophila." Nature **287**(5785): 795-801.

Orsulic, S. and M. Peifer (1996). "An in vivo structure-function study of armadillo, the beta-catenin homologue, reveals both separate and overlapping regions of the protein required for cell adhesion and for wingless signaling." J Cell Biol **134**(5): 1283-1300.

Pai, C. Y., T. S. Kuo, T. J. Jaw, E. Kurant, C. T. Chen, D. A. Bessarab, A. Salzberg and Y. H. Sun (1998). "The Homothorax homeoprotein activates the nuclear localization of another homeoprotein, extradenticle, and suppresses eye development in Drosophila." Genes Dev **12**(3): 435-446.

Papayannopoulos, V., A. Tomlinson, V. M. Panin, C. Rauskolb and K. D. Irvine (1998). "Dorsal-ventral signaling in the Drosophila eye." Science **281**(5385): 2031-2034.

Pastor-Pareja, J. C., F. Grawe, E. Martin-Blanco and A. Garcia-Bellido (2004). "Invasive cell behavior during Drosophila imaginal disc eversion is mediated by the JNK signaling cascade." Dev Cell **7**(3): 387-399.

Peifer, M. and E. Wieschaus (1990). "The segment polarity gene armadillo encodes a functionally modular protein that is the Drosophila homolog of human plakoglobin." Cell **63**(6): 1167-1176.

Pepple, K. L., M. Atkins, K. Venken, K. Wellnitz, M. Harding, B. Frankfort and G. Mardon (2008). "Two-step selection of a single R8 photoreceptor: a bistable loop between senseless and rough locks in R8 fate." Development **135**(24): 4071-4079.

Phillips, R. G., I. J. Roberts, P. W. Ingham and J. R. Whittle (1990). "The Drosophila segment polarity gene patched is involved in a position-signalling mechanism in imaginal discs." Development **110**(1): 105-114.

Pignoni, F. and S. L. Zipursky (1997). "Induction of Drosophila eye development by decapentaplegic." Development **124**(2): 271-278.

Raftery, L. A., M. Sanicola, R. K. Blackman and W. M. Gelbart (1991). "The relationship of decapentaplegic and engrailed expression in Drosophila imaginal disks: do these genes mark the anterior-posterior compartment boundary?" Development **113**(1): 27-33.

Ramirez-Weber, F. A. and T. B. Kornberg (1999). "Cytonemes: cellular processes that project to the principal signaling center in Drosophila imaginal discs." Cell **97**(5): 599-607.

Ready, D. F., T. E. Hanson and S. Benzer (1976). "Development of the Drosophila retina, a neurocrystalline lattice." Dev Biol **53**(2): 217-240.

Reinke, R. and S. L. Zipursky (1988). "Cell-cell interaction in the Drosophila retina: the bride of sevenless gene is required in photoreceptor cell R8 for R7 cell development." Cell **55**(2): 321-330.

Reiter, C., T. Schimansky, Z. Nie and K. F. Fischbach (1996). "Reorganization of membrane contacts prior to apoptosis in the Drosophila retina: the role of the IrreC-rst protein." Development **122**(6): 1931-1940.

Rhee, C. S., M. Sen, D. Lu, C. Wu, L. Leoni, J. Rubin, M. Corr and D. A. Carson (2002). "Wnt and frizzled receptors as potential targets for immunotherapy in head and neck squamous cell carcinomas." Oncogene **21**(43): 6598-6605.

Riggleman, B., P. Schedl and E. Wieschaus (1990). "Spatial expression of the Drosophila segment polarity gene armadillo is posttranscriptionally regulated by wingless." Cell **63**(3): 549-560.

Riggleman, B., E. Wieschaus and P. Schedl (1989). "Molecular analysis of the armadillo locus: uniformly distributed transcripts and a protein with novel internal repeats are associated with a Drosophila segment polarity gene." Genes Dev **3**(1): 96-113.

Roberts, D. B. (2006). "Drosophila melanogaster: the model organism." Entomologia Experimentalis et Applicata **121**(2): 93-103.

Roignant, J. Y. and J. E. Treisman (2009). "Pattern formation in the Drosophila eye disc." Int J Dev Biol **53**(5-6): 795-804.

Rosin-Arbesfeld, R., F. Townsley and M. Bienz (2000). "The APC tumour suppressor has a nuclear export function." Nature **406**(6799): 1009-1012.

Rousset, R., J. A. Mack, K. A. Wharton, Jr., J. D. Axelrod, K. M. Cadigan, M. P. Fish, R. Nusse and M. P. Scott (2001). "Naked cuticle targets dishevelled to antagonize Wnt signal transduction." Genes Dev **15**(6): 658-671.

Roy, S., F. Hsiung and T. B. Kornberg (2011). "Specificity of Drosophila cytonemes for distinct signaling pathways." Science **332**(6027): 354-358.

Rulifson, E. J., C. A. Micchelli, J. D. Axelrod, N. Perrimon and S. S. Blair (1996). "wingless refines its own expression domain on the Drosophila wing margin." Nature **384**(6604): 72-74.

Rylatt, D. B., A. Aitken, T. Bilham, G. D. Condon, N. Embi and P. Cohen (1980). "Glycogen synthase from rabbit skeletal muscle. Amino acid sequence at the sites phosphorylated by glycogen synthase kinase-3, and extension of the N-terminal sequence containing the site phosphorylated by phosphorylase kinase." Eur J Biochem **107**(2): 529-537.

Salzer, C. L., Y. Elias and J. P. Kumar (2010). "The retinal determination gene eyes absent is regulated by the EGF receptor pathway throughout development in Drosophila." Genetics **184**(1): 185-197.

Sato, A., T. Kojima, K. Ui-Tei, Y. Miyata and K. Saigo (1999). "Dfrizzled-3, a new Drosophila Wnt receptor, acting as an attenuator of Wingless signaling in wingless hypomorphic mutants." Development **126**(20): 4421-4430.

Schier, A. F. (2009). "Nodal morphogens." Cold Spring Harb Perspect Biol **1**(5): a003459.

Seto, E. S. and H. J. Bellen (2004). "The ins and outs of Wingless signaling." Trends Cell Biol **14**(1): 45-53.

Sharma, R. P. and V. L. Chopra (1976). "Effect of the Wingless (wg1) mutation on wing and haltere development in Drosophila melanogaster." Dev Biol **48**(2): 461-465.

Siegfried, E., L. A. Perkins, T. M. Capaci and N. Perrimon (1990). "Putative protein kinase product of the Drosophila segment-polarity gene zeste-white3." Nature **345**(6278): 825-829.

Sierra, J., T. Yoshida, C. A. Joazeiro and K. A. Jones (2006). "The APC tumor suppressor counteracts beta-catenin activation and H3K4 methylation at Wnt target genes." Genes Dev **20**(5): 586-600.

Simcox, A. A. and J. H. Sang (1983). "When does determination occur in Drosophila embryos?" Dev Biol **97**(1): 212-221.

Singh, A., J. Chan, J. J. Chern and K. W. Choi (2005). "Genetic interaction of Lobe with its modifiers in dorsoventral patterning and growth of the Drosophila eye." Genetics **171**(1): 169-183.

Singh, A. and K. W. Choi (2003). "Initial state of the Drosophila eye before dorsoventral specification is equivalent to ventral." Development **130**(25): 6351-6360.

Singh, A., M. Tare, O. R. Puli and M. Kango-Singh (2012). "A glimpse into dorso-ventral patterning of the Drosophila eye." Dev Dyn **241**(1): 69-84.

Spemann, H. and H. Mangold (2001). "Induction of embryonic primordia by implantation of organizers from a different species. 1923." Int J Dev Biol **45**(1): 13-38.

Strausfeld, N. J. and J. A. Campos-Ortega (1977). "Vision in insects: pathways possibly underlying neural adaptation and lateral inhibition." Science **195**(4281): 894-897.

Struhl, G. and K. Basler (1993). "Organizing activity of wingless protein in Drosophila." Cell **72**(4): 527-540.

Strutt, D. I., V. Wiersdorff and M. Mlodzik (1995). "Regulation of furrow progression in the Drosophila eye by cAMP-dependent protein kinase A." Nature **373**(6516): 705-709.

Tacchelly-Benites, O., Z. Wang, E. Yang, E. Lee and Y. Ahmed (2013). "Toggling a conformational switch in Wnt/beta-catenin signaling: Regulation of Axin phosphorylation: The phosphorylation state of Axin controls its scaffold function in two Wnt pathway protein complexes." Bioessays.

Takacs, C. M., J. R. Baird, E. G. Hughes, S. S. Kent, H. Benchabane, R. Paik and Y. Ahmed (2008). "Dual positive and negative regulation of wingless signaling by adenomatous polyposis coli." Science **319**(5861): 333-336.

Takemaru, K. I. and R. T. Moon (2000). "The transcriptional coactivator CBP interacts with beta-catenin to activate gene expression." J Cell Biol **149**(2): 249-254.

Tamai, K., M. Semenov, Y. Kato, R. Spokony, C. Liu, Y. Katsuyama, F. Hess, J. P. Saint-Jeannet and X. He (2000). "LDL-receptor-related proteins in Wnt signal transduction." Nature **407**(6803): 530-535.

Tamai, K., X. Zeng, C. Liu, X. Zhang, Y. Harada, Z. Chang and X. He (2004). "A mechanism for Wnt coreceptor activation." Mol Cell **13**(1): 149-156.

Tanaka-Matakatsu, M., T. Uemura, H. Oda, M. Takeichi and S. Hayashi (1996). "Cadherin-mediated cell adhesion and cell motility in Drosophila trachea regulated by the transcription factor Escargot." Development **122**(12): 3697-3705.

Tang, A. H., T. P. Neufeld, E. Kwan and G. M. Rubin (1997). "PHYL acts to down-regulate TTK88, a transcriptional repressor of neuronal cell fates, by a SINA-dependent mechanism." Cell **90**(3): 459-467.

Thomas, B. J., D. A. Gunning, J. Cho and L. Zipursky (1994). "Cell cycle progression in the developing Drosophila eye: roughex encodes a novel protein required for the establishment of G1." Cell **77**(7): 1003-1014.

Tolwinski, N. S., M. Wehrli, A. Rives, N. Erdeniz, S. DiNardo and E. Wieschaus (2003). "Wg/Wnt signal can be transmitted through arrow/LRP5,6 and Axin independently of Zw3/Gsk3beta activity." Dev Cell **4**(3): 407-418.

Tomlinson, A. (1985). "The cellular dynamics of pattern formation in the eye of Drosophila." J Embryol Exp Morphol **89**: 313-331.

TOMLINSON, A. (1988). "Cellular interactions in the developing Drosophila eye." Development **104**(2): 183-193.

Tomlinson, A. (1989). "Short-range positional signals in the developing Drosophila eye." Development **107 Suppl**: 59-63.

Tomlinson, A. (2003). "Patterning the peripheral retina of the fly: decoding a gradient." Dev Cell **5**(5): 799-809.

Tomlinson, A., D. D. Bowtell, E. Hafen and G. M. Rubin (1987). "Localization of the sevenless protein, a putative receptor for positional information, in the eye imaginal disc of Drosophila." Cell **51**(1): 143-150.

Tomlinson, A., Y. E. Mavromatakis and G. Struhl (2011). "Three distinct roles for notch in Drosophila R7 photoreceptor specification." PLoS Biol **9**(8): e1001132.

Tomlinson, A. and D. F. Ready (1986). "Sevenless: a cell-specific homeotic mutation of the Drosophila eye." Science **231**(4736): 400-402.

Tomlinson, A. and D. F. Ready (1987). "Cell fate in the Drosophila ommatidium." Dev Biol **123**(1): 264-275.

Tomlinson, A. and D. F. Ready (1987). "Neuronal differentiation in Drosophila ommatidium." Dev Biol **120**(2): 366-376.

Tomlinson, A. and D. F. Ready (1987). "Neuronal differentiation in the< i> Drosophila</i> ommatidium." Developmental biology **120**(2): 366-376.

Tomlinson, A., W. R. Strapps and J. Heemskerk (1997). "Linking Frizzled and Wnt signaling in Drosophila development." Development **124**(22): 4515-4521.

Tomlinson, A. and G. Struhl (2001). "Delta/Notch and Boss/Sevenless signals act combinatorially to specify the Drosophila R7 photoreceptor." Mol Cell **7**(3): 487-495.

Townsley, F. M., A. Cliffe and M. Bienz (2004). "Pygopus and Legless target Armadillo/beta-catenin to the nucleus to enable its transcriptional co-activator function." Nat Cell Biol **6**(7): 626-633.

Treisman, J. and R. Lang (2002). "Development and evolution of the eye: Fondation des Treilles, September, 2001." Mech Dev **112**(1-2): 3-8.

Treisman, J. E. and U. Heberlein (1998). "Eye development in Drosophila: formation of the eye field and control of differentiation." Curr Top Dev Biol **39**: 119-158.

Treisman, J. E. and G. M. Rubin (1995). "wingless inhibits morphogenetic furrow movement in the Drosophila eye disc." Development **121**(11): 3519-3527.

Tsai, Y. C. and Y. H. Sun (2004). "Long-range effect of upd, a ligand for Jak/STAT pathway, on cell cycle in Drosophila eye development." Genesis **39**(2): 141-153.

Tsuda, L., R. Nagaraj, S. L. Zipursky and U. Banerjee (2002). "An EGFR/Ebi/Sno pathway promotes delta expression by inactivating Su(H)/SMRTER repression during inductive notch signaling." Cell **110**(5): 625-637.

Veeman, M. T., J. D. Axelrod and R. T. Moon (2003). "A second canon. Functions and mechanisms of beta-catenin-independent Wnt signaling." Dev Cell **5**(3): 367-377.

Vinson, C. R. and P. N. Adler (1987). "Directional non-cell autonomy and the transmission of polarity information by the frizzled gene of Drosophila." Nature **329**(6139): 549-551.

Vleminckx, K., R. Kemler and A. Hecht (1999). "The C-terminal transactivation domain of beta-catenin is necessary and sufficient for signaling by the LEF-1/beta-catenin complex in Xenopus laevis." Mech Dev **81**(1-2): 65-74.

Waddington, C. and M. M. Perry (1963). "Inter-retinular fibres in the eyes of< i> Drosophila</i>." Journal of Insect Physiology **9**(4): 475-478.

Waltzer, L. and M. Bienz (1998). "Drosophila CBP represses the transcription factor TCF to antagonize Wingless signalling." Nature **395**(6701): 521-525.

Wang, C. W. and Y. H. Sun (2012). "Segregation of eye and antenna fates maintained by mutual antagonism in Drosophila." Development **139**(18): 3413-3421.

Wehrli, M., S. T. Dougan, K. Caldwell, L. O'Keefe, S. Schwartz, D. Vaizel-Ohayon, E. Schejter, A. Tomlinson and S. DiNardo (2000). "arrow encodes an LDL-receptor-related protein essential for Wingless signalling." Nature **407**(6803): 527-530.

Wehrli, M. and A. Tomlinson (1995). "Epithelial planar polarity in the developing Drosophila eye." Development **121**(8): 2451-2459.

Weigmann, K., R. Klapper, T. Strasser, C. Rickert, G. Technau, H. Jackle, W. Janning and C. Klambt (2003). "FlyMove--a new way to look at development of Drosophila." Trends Genet **19**(6): 310-311.

Wernet, M. F., T. Labhart, F. Baumann, E. O. Mazzoni, F. Pichaud and C. Desplan (2003). "Homothorax switches function of Drosophila photoreceptors from color to polarized light sensors." Cell **115**(3): 267-279.

Whiteley, M., P. D. Noguchi, S. M. Sensabaugh, W. F. Odenwald and J. A. Kassis (1992). "The Drosophila gene escargot encodes a zinc finger motif found in snail-related genes." Mech Dev **36**(3): 117-127.

Wiersdorff, V., T. Lecuit, S. M. Cohen and M. Mlodzik (1996). "Mad acts downstream of Dpp receptors, revealing a differential requirement for dpp signaling in initiation and propagation of morphogenesis in the Drosophila eye." Development **122**(7): 2153-2162.

Willert, K., J. D. Brown, E. Danenberg, A. W. Duncan, I. L. Weissman, T. Reya, J. R. Yates, 3rd and R. Nusse (2003). "Wnt proteins are lipid-modified and can act as stem cell growth factors." Nature **423**(6938): 448-452.

Williams, J. A., S. W. Paddock and S. B. Carroll (1993). "Pattern formation in a secondary field: a hierarchy of regulatory genes subdivides the developing Drosophila wing disc into discrete subregions." Development **117**(2): 571-584.

Williams, J. A., S. W. Paddock, K. Vorwerk and S. B. Carroll (1994). "Organization of wing formation and induction of a wing-patterning gene at the dorsal/ventral compartment boundary." Nature **368**(6469): 299-305.

Wolff, T. and D. F. Ready (1991). "The beginning of pattern formation in the Drosophila compound eye: the morphogenetic furrow and the second mitotic wave." Development **113**(3): 841-850.

Wolff, T. and D. F. Ready (1991). "Cell death in normal and rough eye mutants of Drosophila." Development **113**(3): 825-839.

Wolpert, L. (1971). "Positional information and pattern formation." Curr Top Dev Biol **6**(6): 183-224.

Wong, H. C., A. Bourdelas, A. Krauss, H. J. Lee, Y. Shao, D. Wu, M. Mlodzik, D. L. Shi and J. Zheng (2003). "Direct binding of the PDZ domain of Dishevelled to a conserved internal sequence in the C-terminal region of Frizzled." Mol Cell **12**(5): 1251-1260.

Wu, J., A. Jenny, I. Mirkovic and M. Mlodzik (2008). "Frizzled-Dishevelled signaling specificity outcome can be modulated by Diego in Drosophila." Mech Dev **125**(1-2): 30-42.

Yagi, R., F. Mayer and K. Basler (2010). "Refined LexA transactivators and their use in combination with the Drosophila Gal4 system." Proceedings of the National Academy of Sciences **107**(37): 16166-16171.

Yamamoto, H., S. Kishida, M. Kishida, S. Ikeda, S. Takada and A. Kikuchi (1999). "Phosphorylation of axin, a Wnt signal negative regulator, by glycogen synthase kinase-3beta regulates its stability." J Biol Chem **274**(16): 10681-10684.

Yin, H., X. Wen, H. Qiu and T. Zhou (2013). "Cooperative Diffusion Leads to a Robust Morphogen Gradient." CHINESE JOURNAL OF PHYSICS **51**(2).

Yu, S. R., M. Burkhardt, M. Nowak, J. Ries, Z. Petrasek, S. Scholpp, P. Schwille and M. Brand (2009). "Fgf8 morphogen gradient forms by a source-sink mechanism with freely diffusing molecules." Nature **461**(7263): 533-536.

Zecca, M., K. Basler and G. Struhl (1996). "Direct and long-range action of a wingless morphogen gradient." Cell **87**(5): 833-844.

Zecca, M. and G. Struhl (2007). "Control of Drosophila wing growth by the vestigial quadrant enhancer." Development **134**(16): 3011-3020.

Zecca, M. and G. Struhl (2007). "Recruitment of cells into the Drosophila wing primordium by a feed-forward circuit of vestigial autoregulation." Development **134**(16): 3001-3010.

Zeng, L., F. Fagotto, T. Zhang, W. Hsu, T. J. Vasicek, W. L. Perry, 3rd, J. J. Lee, S. M. Tilghman, B. M. Gumbiner and F. Costantini (1997). "The mouse Fused locus encodes Axin, an inhibitor of the Wnt signaling pathway that regulates embryonic axis formation." Cell **90**(1): 181-192.

Zeng, X., K. Tamai, B. Doble, S. Li, H. Huang, R. Habas, H. Okamura, J. Woodgett and X. He (2005). "A dual-kinase mechanism for Wnt co-receptor phosphorylation and activation." Nature **438**(7069): 873-877.

Zimmerman, J. E., Q. T. Bui, H. Liu and N. M. Bonini (2000). "Molecular genetic analysis of Drosophila eyes absent mutants reveals an eye enhancer element." Genetics **154**(1): 237-246.

2. Materials and methods

1. Drosophila genetics

Stocks were maintained at 18°C and 25°C. All crosses and staging were performed at 25°C. Pupal development was defined as number of hours After Puparium Formation (APF), with the white pre pupa stage defined as 0hrsAPF.

Stocks used	**Stock information**
General stocks	
D-APC^{Q8}	(Ahmed, Hayashi et al. 1998)
hs-flp	Lab stock
GMR-wg	(Wehrli and Tomlinson 1998)
GMR-flp	Lab stock
GMR-p35	(Hay, Wolff et al. 1994)
GMR-Hid	(Bergmann, Agapite et al. 1998)
Tub-α1>w+>wg	(Wehrli and Tomlinson 1998)
Canton S (wild type flies)	Lab stock
LacZ lines	
Esg-LacZ	Lab stock
Wg-lacZ	Lab stock
UAS lines	
UAS-deGFP	(Lieber, Kidd et al. 2011)
UAS-GFP	(Johnston and Sanders 2003)
UAS-Axn-GFP	(Cliffe, Hamada et al. 2003)

UAS-Wg	(Zecca, Basler et al. 1996)
UAS-Nrt-Wg	(Zecca, Basler et al. 1996)
*UAS-Arm**	(Zecca, Basler et al. 1996)
UAS-Esg	Lab stock
UAS-Esg-RNAi	TRiP stock (BL# 28514)
UAS-Wg-RNAi	Gift from G. Struhl
UAS-PanRNAi	VDRC stock (#3014)
Gal4 Driver lines	
GMR-Gal4	(Hay, Wolff et al. 1994)
Notum-Gal4	(Gerlitz and Basler 2002)
Pros-Gal4	(Hayashi, Xu et al. 2008)
Otd-Gal4	(Sprecher, Pichaud et al. 2007)
LongGMR-Gal4	(Wernet, Labhart et al. 2003)
Elav(C155)-Gal4	(Ahmed, Hayashi et al. 1998)
Elav(II)Gal4	Gift from R. Axel
Genotypes generated for the experiments	
yw122; sp/CyO; GMR-Wg.UAS-Pan-RNAi/TM6B	
yw122; UAS-Arm/CyO; Pros-Gal4/TM6B*	
yw122; UAS-Arm;GMR-Gal4/ SM6-TM6B*	
yw122;Esg-lacZ/CyO;Pros-Gal4.UAS-EsgRNAi/TM2	

yw122; Tub-α1>w+>wg/CyO; GMR-flp/TM2	
yw122; UAS-Axn-GFP/CyO; Otd-Gal4/TM2	
yw122; UAS-deGFP/CyO; Otd-Gal/TM6B	

2. Adult eye sectioning protocol

   Adult heads were dissected in PBS, and fixed in 2.5% glutaraldehyde for 45 min on ice, and then dehydrated through a graded alcohol series (30%EtOH-100%EtOH, 5 min each). After 100% ethanol the eyes were transferred to propylene oxide for 15 min and then left in a 50% mixture of propylene oxide and Durcupan resin for 30 min. They were then transferred to pure resin, and further necessary dissections performed before embedding and polymerization. The embedded retinas were sectioned tangentially for analysis of the main body of the eye, and sideways along the A/P and D/V axes of the eye for peripheral analysis. Each section is 0.5 μm in thickness. The adult eye sections were imaged on a light microscope, and edited using Adobe Photoshop software.

3. Immunofluorescence

   - Pupal eye dissection protocol

   Pupal eye discs were dissected in PBS (Phosphate buffered saline) and the extracted eye discs (with optic lobes still attached) were transferred to PBS on ice. Discs were fixed in 4% FA (formaldehyde) in PBS on ice for 45min and then washed in PBS (1X) for 5 min. The fixed eye discs were rinsed in 0.05% PBT (PBS 1X+ 0.05% Triton-X100). The tissue was then incubated with the primary antibody mix (diluted in PBT) overnight at

4°C. The following day the discs were washed with PBT (3 times, 15 min each) and then incubated with the secondary antibody mix (diluted with PBT) for 3 hr at room temperature. After incubation, the eye discs were washed in PBT and the optic lobes were dissected out. The flat pupal retinas were mounted on slides in Vectashield. The slides were stored at 4°C.

- Larval eye dissection protocol

Larval eye discs were dissected in PBS (Phosphate buffered saline) and were fixed in 4% FA (formaldehyde) in PBS for 20min at room temperature. The discs were then washed twice in PBS (1X) for 5 min each. The fixed eye discs were rinsed in 0.1% PBT (PBS 1X+ 0.1% Triton-X100). The tissue was then incubated with the primary antibody mix (diluted in PBT) overnight at 4°C (or at room temperature for 2 hours). The following day the discs were washed with PBT (5 times, 10 min each) and then incubated with the secondary antibody mix (diluted with PBT) for 2 hr at room temperature. After incubation, the eye discs were washed in PBT and the eye discs were mounted on slides in Vectashield. The slides were stored at 4°C.

- Image analysis – The immunofluorescence images were taken on a Leica SP5 confocal microscope, and edited using Adobe Photoshop software.

4. Antibodies

The following antibodies were used for the various experiments: mouse anti-Cut (1:20), rat anti-Elav (1:50), Mouse anti-Wg (1:20) (all three from Developmental Studies Hybridoma Bank), rabbit anti-cleaved Caspase-3 (1:200) (Cell Signaling Technologies), rabbit anti-β-gal (1:1000) (Cappell), rabbit anti-GFP (1:500), mouse anti-GFP (1:500) (both from Molecular Probes), guinea pig anti-Hth (1:200) (gift from R. Mann), mouse

anti-Svp (1:20) (gift from Y. Hiromi). Secondary antibodies used were Alexa Fluor 488, 555, and 647 (Molecular Probes). DAPI was used at 1:1000.

Ahmed, Y., S. Hayashi, A. Levine and E. Wieschaus (1998). "Regulation of armadillo by a Drosophila APC inhibits neuronal apoptosis during retinal development." Cell **93**(7): 1171-1182.

Bergmann, A., J. Agapite, K. McCall and H. Steller (1998). "The Drosophila gene hid is a direct molecular target of Ras-dependent survival signaling." Cell **95**(3): 331-341.

Cliffe, A., F. Hamada and M. Bienz (2003). "A role of Dishevelled in relocating Axin to the plasma membrane during wingless signaling." Curr Biol **13**(11): 960-966.

Gerlitz, O. and K. Basler (2002). "Wingful, an extracellular feedback inhibitor of Wingless." Genes Dev **16**(9): 1055-1059.

Hay, B. A., T. Wolff and G. M. Rubin (1994). "Expression of baculovirus P35 prevents cell death in Drosophila." Development **120**(8): 2121-2129.

Hayashi, T., C. Xu and R. W. Carthew (2008). "Cell-type-specific transcription of prospero is controlled by combinatorial signaling in the Drosophila eye." Development **135**(16): 2787-2796.

Johnston, L. A. and A. L. Sanders (2003). "Wingless promotes cell survival but constrains growth during Drosophila wing development." Nat Cell Biol **5**(9): 827-833.

Lieber, T., S. Kidd and G. Struhl (2011). "DSL-Notch signaling in the Drosophila brain in response to olfactory stimulation." Neuron **69**(3): 468-481.

Sprecher, S. G., F. Pichaud and C. Desplan (2007). "Adult and larval photoreceptors use different mechanisms to specify the same Rhodopsin fates." Genes Dev **21**(17): 2182-2195.

Wehrli, M. and A. Tomlinson (1998). "Independent regulation of anterior/posterior and equatorial/polar polarity in the Drosophila eye; evidence for the involvement of Wnt signaling in the equatorial/polar axis." Development **125**(8): 1421-1432.

Wernet, M. F., T. Labhart, F. Baumann, E. O. Mazzoni, F. Pichaud and C. Desplan (2003). "Homothorax switches function of Drosophila photoreceptors from color to polarized light sensors." Cell **115**(3): 267-279.

Zecca, M., K. Basler and G. Struhl (1996). "Direct and long-range action of a wingless morphogen gradient." Cell **87**(5): 833-844.

3. Results

Objective 1: Understanding pigment rim formation at the periphery

The *Drosophila* compound eye is a repetitive array of ommatidia arranged in a precisely ordered lattice (figure 1A). The ommatidia consist of four cone cells, eight photoreceptors and two primary pigment cells (figure 1C). These ommatidia are nestled in a honeycomb lattice made up of secondary and tertiary pigment cells (Waddington and Perry 1960, Ready, Hanson et al. 1976). The mechanosensory bristles are present at the alternate vertices of this hexagonal pigment cell lattice (Cagan and Ready 1989). The lattice is pruned during pupal development to form the precise spatial array by multiple rounds of apoptosis to remove excess inter ommatidial pigment cells. In addition to this, there is a mid-pupal round of apoptosis at the periphery of the retina, which causes removal of the outermost row of ommatidia (Wolff and Ready 1991, Hay, Wassarman et al. 1995).

During third larval instar and pupal development, the presumptive head capsule (HC) tissue surrounding the retina expresses Wg (Ma and Moses 1995, Treisman and Rubin 1995), and this diffusing Wg forms a gradient at the periphery of the pupal eye (Wehrli and Tomlinson 1998, Tomlinson 2003) (Figure 2 D). This gradient is interpreted to form the peripheral retinal specializations: low levels of Wg prevent bristle formation (Cadigan, Jou et al. 2002); intermediate levels of Wg lead to formation of plane polarized light detectors, called the Dorsal Rim Ommatidia (DRO) (Wernet, Labhart et al. 2003); and high levels of Wg lead to the formation of the pigment rim (Tomlinson 2003) (Figure 2A, D). Wg signaling from the surrounding HC induces its own expression in the cone

cells of the peripheral ommatidia at 32hrs After Puparium Formation (APF), followed by the mid-pupal apoptosis of these ommatidia at 42hrsAPF. These peripheral ommatidia are reported to be frequently incomplete and lacking proper connections to the optic lobes, hence they are culled via apoptosis (Lin, Rogulja et al. 2004). Following the peripheral apoptosis, the secondary/tertiary pigment cells surrounding these outermost ommatidia coalesce to form the pigment rim (Tomlinson 2003). It was later shown that this ommatidial Wg (henceforth called the secondary Wg - 2° Wg) expression was restricted to a subset of the ommatidial cells destined to die – namely, the cone cells (Lim and Tomlinson 2006). It was also shown that these peripheral ommatidia express the Snail class family of zinc-finger transcription factors (Escargot, Snail and Worniu) and Notum, an α/β hydrolase that acts to restrict Wg diffusion, in their cone cells and in the surrounding secondary/tertiary pigment cells. Furthermore, it was shown that these responses were required for the peripheral ommatidial apoptosis (Lim and Tomlinson 2006).

Based on these observations, we hypothesized that in response to the HC-derived Wg, the cone cells of the peripheral ommatidia, in addition to expressing the known molecular responses, send out a non-autonomous 'death signal' to lead to apoptosis of the associated ommatidial cells – the photoreceptors and the 1° pigment cells. We wished to understand the mechanism of this Wg – induced death of different ommatidial cell types, leading to the formation of the pigment rim.

To do so, the first set of experiments we conducted were designed to understand the sequence of events at the periphery of the eye leading to the formation of the adult eye pigment rim.

1.1 Characterization of pupal developmental events in a wild type eye periphery

By early pupation (~ 20hrsAPF), all the ommatidial cells are assembled and the retinal lattice is established (Cagan and Ready 1989). During mid-pupal stages, the cells undergo various morphological changes associated with their specialized functions. The cone cells are drawn out into thin inter-retinular fibers as the retina deepens (Waddington and Perry 1963, Cagan and Ready 1989), but their nuclei maintain apical positions overlying the photoreceptors (Figure 3A). The photoreceptors orient themselves in the characteristic trapezoid pattern, and their apical membranes undergo transformation to microvillar structures called rhabdomeres (Perry 1968), which continue to elongate during pupal development (Figure 3B). The 1° pigment cells are found apically alongside the cone cells (Figure 3A). The secondary/tertiary pigment cells are present at the basal regions of the retina, insulating the ommatidia (Cagan and Ready 1989) (Figure 3C).

At the periphery at 32hrsAPF, the cone cells of the outermost ommatidia express Wg (as assessed by the expression of Wg protein (Figure 3D), Escargot (Esg), (as assessed by the expression of the transcriptional reporter *Esg-LacZ*)(Figure 3E) and Notum (as assessed by GFP expression from a *UAS-GFP* transgene driven by the *Notum-Gal4* driver line)(Figure 3F) (Lim and Tomlinson 2006). Henceforth, we shall be using Esg as the representative of the Snail class proteins, and when we mention

Esg, we are implying that we are accounting for all three proteins – Snail, Escargot and Worniu. As these Snail class proteins have been reported to be functionally redundant in various developmental processes including the peripheral patterning of the eye (Boulay, Dennefeld et al. 1987, Whiteley, Noguchi et al. 1992, Ashraf, Hu et al. 1999, Lim and Tomlinson 2006), we infer that all three proteins will show identical responses. At 36hrsAPF, the cone cell nuclei at the periphery lose their apical position and collapse to the level of the photoreceptors (Figure 3G). At 42hrsAPF, the outermost row of ommatidia (cone cells, photoreceptors and 1° pigment cells) undergoes apoptosis (as evidenced by the presence of cleaved caspase-3, a marker for apoptosis)(Figure 3H) (Wolff and Ready 1991, Yu, Yoo et al. 2002, Fan and Bergmann 2010). The surrounding 2°/3° pigment cells coalesce during later pupal development to form the pigment rim (Tomlinson 2003), as seen in the adult eye peripheral sections (Figure 3I).

In summary, the peripheral events leading to pigment rim formation are: expression of Wg, Esg and Notum in the cone cells at 32hrsAPF, collapse of the cone cells at 36hrsAPF and apoptosis of the entire ommatidium at 42hrsAPF. We have not looked at the effects on the 1° pigment cells at these stages owing to the lack of good tools for their analysis.

In order to understand how Wg accomplishes this cascade of events to form the pigment rim; the first hypothesis we formulated was that the peripheral ommatidia are somehow 'primed' to respond to high levels of Wg signaling. If this is true, then, upon being subjected to high levels of Wg signaling, the ommatidia in the main body of the eye

should not respond in a similar manner as the peripheral ommatidia. To test this hypothesis, we utilized a transgene which causes expression of Wg at high levels in all the cells of the retina, *GMR-wg* (Wehrli and Tomlinson 1998). This transgene drives Wg cDNA expression from the Glass Multimer Repeats (GMR) enhancer element, which leads to expression in all the cells of the eye posterior to the morphogenetic furrow (Moses and Rubin 1991). This ensures that we are not affecting early Wg-dependent larval developmental events, but are actually assessing the effects of ectopic expression of Wg in the pupal stages. The adult eyes of *GMR-Wg* genotype flies are much smaller as compared to a wild type eye, and contain only pigment cells (Tomlinson 2003) (Figure 3). Externally the eye surface is bald and glazed. The HC region surrounding the eye is slightly enlarged in a *GMR-Wg* eye as compared to a wild type eye (figure 4A-4B). This is probably due to the diffusion of the ectopic Wg ahead of, and to the sides of the morphogenetic furrow, thus restricting the retinal field to a smaller area while expanding the HC region (Treisman and Rubin 1995). Sections through these *GMR-Wg* eyes show that the retina lacks ommatidial structures and pigment cell lattice; instead the eye tissue resembles the pigment rim (Figure 4C-D'). These observations suggest that the entire eye responds similarly to high levels of Wg, but wished to confirm that the *GMR-Wg* eyes underwent the same pupal developmental events as the peripheral ommatidia in a wild type eye.

1.2 Effect of ectopic Wg expression on the pupal developmental stages of the eye

In *GMR-Wg* pupal eyes, the ommatidia undergo wild type development up until 32hrsAPF (figure 5 A-A'). The cone cell nuclei lie apically over the photoreceptors as

the retina deepens. However, at 32hrsAPF, we observe that similar to the peripheral cone cells of a wild-type retina; all the cone cells express Wg and Esg (Figure 5B-C). This expression was monitored using the transcriptional reporter lines *Wg-lacZ* and *Esg-lacZ* in a *GMR-Wg* background. At 36hrs APF, all the cone cells of the retina collapse to the level of the photoreceptors (Figure 5 D-D'). By 39hrsAPF, cone cells begin to undergo apoptosis(Figure 5E), and by 42hrsAPF, all the ommatidial cells in the retina – including cone cells, photoreceptors and 1° pigment cells, undergo apoptosis (Figure 5F). This apoptosis is evidenced by the presence of cleaved caspase 3 (Figure 5F).

Taken together, the above data suggest that in response to high levels of ectopic *Wg* signaling, all the ommatidia of the retina respond in a similar manner, leading to timed mid-pupal apoptosis of these ommatidia, and formation of pigment rim like tissue. These data also suggest that we can utilize the *GMR-wg* transgene as a tool to transform the entire eye to a pigment-rim like tissue, henceforth referred to as the 'pseudo periphery', which can be further analyzed to understand the mechanism by which Wg accomplishes the death of the entire ommatidium.

The previous set of experiments suggests that ectopic expression of Wg leads to ommatidial apoptosis. Then, by corollary, removal of Wg transduction should prevent the death of the ommatidia. To test this hypothesis, we used Axin, a downstream member of the Wg signaling cascade. Axin (Axn) is a part of the Armadillo-degradation-complex, and acts to recruit Armadillo (Arm) (the fly β-catenin) to this complex and enhances its phosphorylation (Hart, de los Santos et al. 1998, Ikeda, Kishida et al. 1998). This

phosphorylation targets Arm for degradation, thus preventing it from activating the transcription of Wg target genes. Ectopic expression of Axn sequesters Arm at the plasma membrane, thus blocking downstream Wg signal transduction in a cell autonomous manner (Mendoza-Topaz, Mieszczanek et al. 2011). Using the GAL4-UAS system (Brand and Perrimon 1993), we drive the expression of *UAS-Axn-GFP* (Cliffe, Hamada et al. 2003) transgene in all the cells of the eye with the *GMR-Gal4* driver line (Hay, Wolff et al. 1994).

1.3 Effect of blocking Wg transduction on pupal development of the eye

To assess the effect of blocking Wg signal transduction in the *GMR-wg* induced pseudo periphery, we looked at the eyes of *GMR-Wg; GMR-Gal4; UAS-Axn-GFP* flies. The adult eyes of this genotype look completely wild type (Figure 6G). Sections through the eyes show a wild type array of ommatidia, indicating that the *GMR-Wg* induced pseudo periphery phenotype has been rescued(Figure 6H-J). These results also indicate that we can utilize Axn as an effective tool to block Wg transduction in a cell autonomous manner. To test if these results are replicated at the real periphery, we looked at the periphery of the eyes of *GMR-Gal4; UAS-Axn-GFP* flies. The adult eyes show the presence of small lenses adjacent to the HC, indicative of the incomplete peripheral ommatidia (Figure 6L). Sections through the periphery of the adult eyes show the presence of small ommatidia closer to the HC, and a reduced pigment rim region(Figure 6L'), indicating that the peripheral apoptotic response has been abolished.

These data suggest that blocking Wg signal transduction is sufficient to rescue the peripheral ommatidial apoptosis. Pupal developmental analysis remains to be done in order to confirm that the Wg-dependent peripheral molecular responses are abolished in these genetic backgrounds.

Now that we have established that high levels of Wg signaling lead to the apoptosis of the ommatidia of the entire eye, our next question was – does prevention of apoptosis rescue the *GMR-Wg* pseudo periphery phenotype? If this were the case, then upon blocking the apoptosis pathway in a *GMR-Wg* eye, we should see a wild-type eye. We would also expect the eyes to be bald, and all the dorsal ommatidia to be specialized like the DRO. This phenotype would indicate that we have abolished only the high threshold death response to Wg by blocking apoptosis, but the intermediate and low level responses are still present.

1.4 Effect of blocking apoptosis on *GMR-Wg* induced pseudo periphery

The baculovirus protein p35 has been reported to act as an inhibitor of apoptosis. This inhibition is mediated by binding to, and preventing caspase activation (Clem, Fechheimer et al. 1991). Expressing p35 throughout the eye using *GMR-p35* transgene has been reported to block apoptosis (Hay, Wolff et al. 1994). The *hid* (head involution defective) gene is a key mediator of the apoptotic cascade (Grether, Abrams et al. 1995). Hid induces apoptosis via cleavage and activation of caspases (Wang, Hawkins et al. 1999, Goyal, McCall et al. 2000). *GMR-Hid* flies express Hid in all the cells of the eye (Bergmann, Agapite et al. 1998). The adult eyes of this

genotype are extremely small and the retinal field is reduced to slits. There are no lenses or bristles on the surface of the eye (Bergmann, Agapite et al. 1998). Sections through these eyes show a complete absence of ommatidial structures and very few pigment cells remain (Figure 7A). We also observe clumps of golden and brown extracellular pigments, which we infer was extruded from the dying cells(Figure 7A). To assess the effect of blocking apoptosis in this genetic background, we looked at the eyes of *GMR-Hid; GMR-p35* flies. The adult eyes of these flies look completely wild type. The lenses and bristles on the surface are normal. Sections through these eyes show a normal array of ommatidia, albeit there are additional inter-ommatidial secondary/tertiary pigment cells (Figure 7B). We infer that these extra cells are present because the prevention of apoptosis also prevented the late pupal trimming of supernumerary pigment cells to achieve the uniform pigment cell lattice structure. However, this set of results indicates that *GMR-p35* is an effective tool to block apoptosis in the retina.

To check what happens to the pseudo periphery upon blocking apoptosis, we looked at the eyes of *GMR-Wg; GMR-p35* flies. The size of the adult eyes of this genotype is similar to that of wild type eyes (Figure 7D). However, externally the eyes lack lenses and bristles. Sections through these eyes show necrosis of the lenses (Figure 7E), and lots of degenerated, stunted ommatidia are seen in the apical sections (Figure 7F). Deeper sections show the presence of pigment rim like tissue, indicating that the photoreceptors do not extend the depth of the retina (Figure 7G). These data indicate

the while prevention of apoptosis restores the size of the eye to wild type proportions; chronic exposure to high levels of Wg causes degeneration of the ommatidia.

Conclusions: Based on the data presented so far, we conclude that in response to Wg signaling from the HC, the cone cells of the peripheral ommatidia express Wg, Esg and Notum. This expression pattern is followed by the collapse of cone cells and the apoptosis of the peripheral ommatidia in a precisely timed manner. Furthermore, we showed that ectopic expression of Wg at high levels throughout the eye transforms the entire eye field to a pigment rim-like pseudo periphery. This pseudo periphery phenocopies the peripheral Wg dependent molecular and morphological responses, thus it can be used to analyze the mechanism of this ommatidial apoptosis. We also showed that prevention of apoptosis can rescue the ommatidia but they are degenerated owing to chronic exposure to Wg.

In the following sections, we shall deal with the strategies we employed to understand how Wg signaling leads to the concerted apoptosis of the different ommatidial cell types, namely the cone cells, photoreceptors and the primary pigment cells.

Objective 2: Understanding the role of cone cells in the Wingless mediated peripheral apoptosis

In response to high levels of Wg signaling from the HC, the primary response of the outermost row of ommatidia entails the expression of Wg, Esg and Notum in their cone cells; and this expression is followed by the apoptosis of the entire ommatidium (Lim and Tomlinson 2006). In the previous section, we showed that this apoptotic cascade occurs as a timed sequence of events – expression of Wg, Esg and Notum at 32hrsAPF, collapse of the cone cells at 36hrsAPF and apoptosis of the ommatidia at 42hrsAPF (Figure 3). As the initial response of the peripheral ommatidia appears to be the Wg induced gene expression in the cone cells at 32hrsAPF followed by their collapse; we wished to examine the effects of manipulating the cone cells' response to Wg signaling, on the peripheral apoptosis.

In order to accomplish cone-cell specific expression of the desired transgenes, we decided to use the *Pros-Gal4* transgene (Xu, Kauffmann et al. 2000, Hayashi, Xu et al. 2008). This driver line is expressed strongly in all the cone cells throughout pupal development, as evidenced by expression from a *UAS-GFP* transgene (Figure 8). Although this Gal4 line is also expressed in the R7 photoreceptor cell during larval development (Xu, Kauffmann et al. 2000, Hayashi, Xu et al. 2008), the expression in the R7 cell is not significant for our experiments as we see similar results in *sevenless* mutant flies, which lack the R7 cell. Presently this line is the most specific driver line available

for cone cell expression, and we will be using it for the experiments described in the following sections.

2.1 Effect of blocking Wg transduction in the cone cells

We wished to investigate the role of the cone cell specific gene expression and collapse in the Wg induced apoptotic cascade in the eye. We hypothesized that if this peripheral apoptosis follows a hierarchy of steps, preventing the first step would prevent the occurrence of the subsequent steps; and conversely, triggering the initial steps would ensure the occurrence of the later steps. Hence the question we sought to address was: Would the prevention of Wg signaling in the cone cells prevent the apoptosis of the ommatidia? In order to accomplish cone cell specific blocking of downstream Wg signal transduction, we utilized *UAS-Axn-GFP* driven by the *Pros-Gal4* driver line. As described previously, Axn is a member of the Wg signaling cascade that targets Arm for degradation, and thus prevents transcription of Wg target genes.

Effect of blocking Wg signal transduction in the cone cells in the pseudo periphery

We know that *GMR-Wg* induces the formation of pigment-rim like pseudo periphery, which phenocopies the molecular and morphological responses of the periphery, hence we decided to use this pseudo periphery to assess the effects of blocking Wg transduction in the cone cells. To do so, we examined the eyes of *GMR-Wg; Pros-Gal4; UAS-Axn-GFP* flies. In these eyes, all the cells of the retina are experiencing high levels of *Wg* signaling except the cone cells. It is important to note that the GMR enhancer element is directly fused to the *Wg* cDNA, hence it will not drive the

expression of the *UAS-Axn-GFP* transgene. The adult eyes of this genotype show a significant rescue of the lenses, and a partial rescue of the size of the whole eye (Figure 9 F-H') as compared to a wild-type eye (Figure 9 A). There are no bristles present in the eye (Figure 9 F'). The sections through the adult eyes show lenses that are wild type in appearance (Figure 9 G), although the surrounding pigment cells are abnormal in appearance as compared to the wild type (Figure 9 B). Deeper sections show that the entire retina is devoid of photoreceptors (Figure 9H). The secondary/tertiary (2°/3°) pigment cell lattice structure is not evident; instead we see a few disorganized groups of pigment cells adjacent to the remnants of the ommatidia (Figure 9H).

As an additional way of blocking Wg transduction, we used an RNAi against Pangolin (Pan), which is the fly homolog of TCF/LEF transcription factors. Pan is a part of the transcriptional co-activator complex, which leads to the activation of Wg target gene transcription upon binding to Arm. In the absence of Pan, the transcriptional activator complex is not formed and thus Wg signal transduction is blocked. When we examined the eyes of *UAS-Axn-GFP; GMR-Wg, UAS-Pan-RNAi / Pros-Gal4* flies, we found that there was no significant enhancement in the degree of prevention of apoptosis of ommatidia in the pseudo periphery (Figure 9 I-K). The analysis of *GMR-Wg; Pros-Gal4; UAS-Pan-RNAi* eyes showed a similar phenotype, albeit slightly weaker, which we attributed to insufficient knockdown of *Pan* by the *UAS-Pan-RNAi* transgene. These results suggest that the apoptosis and pseudo-periphery phenotype caused by *GMR-Wg* can only be partially rescued (lenses

rescued but photoreceptors are still absent) by blocking Wg transduction in the cone cells. Now, we decided to assess if this partial rescue also occurs at the real periphery of the eye.

Effect of blocking Wg transduction in the cone cells at the periphery

To examine the effects of blocking the cone cells' Wg response at the periphery, we examined the eyes of *Pros-Gal4; UAS-Axn-GFP* flies. The sections through the adult eyes indicate the presence of small lenses adjacent to the HC (Figure 10 B). These are suggestive of the small lenses secreted by the peripheral ommatidia which are frequently incomplete (Lin, Rogulja et al. 2004). Deeper sections through these eyes show the absence of photoreceptors underneath these peripheral lenses (Figure 10 B'). In order to better understand this partial rescue phenotype, we examined the pupal development of these eyes at the relevant stages. We wished to examine the molecular and morphological responses of the peripheral ommatidia, and compare them to the wild type scenario, to interpret the adult eye phenotype.

When we examined the pupal retinas of *Pros-Gal4; UAS-Axn-GFP* at 32hrsAPF, we see that there is extremely reduced Wg expression in the peripheral cone cells, as assessed by anti-Wg antibody staining (Figure 10D). At 36hrsAPF, unlike their wild type counterparts (Figure 9C), the peripheral cone cells fail to collapse (Figure 10 E-F'). At 42hrsAPF, the peripheral cone cells are present at their normal apical position, while the underlying photoreceptors undergo apoptosis in a manner similar to peripheral ommatidia in a wild type eye (Figure 10G-H').

Taken together, the data presented above suggest that while removing Wg signal transduction in the cone cells can restore the wild type appearance of the lenses and prevent the cone cell apoptosis, it is not sufficient to prevent the Wg induced photoreceptor apoptosis. This indicates that while the cone cells might be the primary responders to the Wg signal from the HC, the photoreceptors and pigment cells must also be involved in the ommatidial response to Wg.

2.2 Effect of activating Wg signal transduction in the cone cells

In response to the Wg signal from the HC, the cone cells are the primary responders. In the previous section we showed data suggesting that preventing cone cells' apoptosis is insufficient to prevent the apoptosis of the rest of the ommatidia, thereby indicating that there is a combinatorial response to the Wg signal. We now wished to assess the degree of response elicited by the cone cells in response to Wg, and what effect, if any, it has on the other cells of the ommatidia. Furthermore, if the cone cells are indeed the primary responders, is triggering the cone cell response sufficient to activate the downstream components of the cascade?

As we showed in Section 1 of this chapter, the entire eye behaves similarly to the periphery upon being subjected to chronic high levels of Wg signaling. Therefore, all the cone cells should also behave in a manner similar to the peripheral cone cells upon being subjected to high levels of Wg signaling. To test this hypothesis, we again utilize the *Pros-Gal4* driver line to cause activation of Wg signaling in the cone cells. When we drive expression of Wg in the cone cells using a *UAS-Wg* transgene (Zecca, Basler et al. 1996) (*Pros-Gal4; UAS-Wg*), we see an adult eye which looks similar to

a *GMR-Wg* eye (Figure 12 C). In order to avoid the non-autonomous effects of using *UAS-Wg*, we used a modified form of Wg, which is tethered to the cell membrane and is unable to be secreted. We utilized this *UAS-Nrt-Wg* (Zecca, Basler et al. 1996) transgene to assess the effects of Wg expression restricted to the cone cells. When we examined *Pros-Gal4; UAS-Nrt-Wg* retinas, the eyes resemble *GMR-Wg* eyes (Figure 12 D). A possible explanation for this phenotype might be that the cone cell membranes are in contact with the rest of the cells of the ommatidia – namely the photoreceptors and the pigment cells. We infer that in this genotype, the other cell types are able to sense the Wg tethered to the cone cell membranes, thereby making it difficult to assess independent contribution of different cell types to the death phenotype.

Therefore, in order to cause cell autonomous activation of the signaling pathway, we utilized a downstream member of the Wg pathway: Arm- the fly β-catenin. As described in the Introduction chapter, in the absence of Wg, Arm is targeted for phosphorylation and degradation. In the presence of Wg, phosphorylation of Arm is prevented, thus stabilizing it in the cytoplasm and allowing its accumulation. Arm translocates to the nucleus and leads to the transcription of Wg target genes. A modified version of Arm, referred to as Activated Armadillo (Arm*), carries an N-terminal deletion which prevents it from being phosphorylated and subsequently degraded, thus rendering the protein constitutively active (Zecca, Basler et al. 1996). Ectopic expression of this Arm* in a cell leads to chronic activation of Wg signaling

in a cell autonomous manner. We therefore used the *UAS-Arm** transgene for our cell autonomous ectopic activation experiments.

To test the efficacy of this method, we checked if the expression of Arm* throughout the retina reproduces the *GMR-Wg* phenotype. If it does, then this would indicate that the *UAS-Arm** transgene causes a strong enough induction of Wg signaling in the cells for us to assess peripheral patterning events. When we checked *GMR-Gal4; UAS-Arm** flies, the eyes resemble *GMR-Wg* eyes, although they are slightly larger in size (Figure 11A-B). Sections through the adult eyes show that *GMR-Gal4; UAS-Arm** phenocopies the *GMR-Wg* eye phenotype (Figure 11C-D), thus confirming that this approach could be utilized for cell specific activation of *Wg* signaling.

Cone cell specific activation of Wg signaling

To cause cell autonomous activation of Wg signaling in the cone cells, we used *Pros-Gal4* to drive the expression of *UAS-Arm**. Theses adult eyes are smaller in size than a wild-type eye (Figure 12 E) Bristles are present on the surface; however instead of normal lenses we see few abnormal, fused lenses on the surface of the retina (Figure 12 E'). Apical sections through these eyes show the presence of a large number of ommatidia bearing incomplete sets of photoreceptors (Figure 12 E''). Unlike the precise trapezoid arrangement of wild type photoreceptors, these photoreceptors are distorted, with twisted rhabdomeres(Figure 12 E''). The inter-ommatidial pigment cell lattice is also disarrayed (Figure 12 E''). Deeper sections show that these ommatidia are stunted i.e. most of the photoreceptors fail to extend the depth of the retina (Figure 12 E'''). Occasionally these eyes show an extreme phenotype with a

complete degeneration of the photoreceptors, although remnants of the pigment cell lattice are still evident (Figure 12 F).

Interestingly, when we look at the peripheral region of the *Pros-Gal4; UAS-Arm** eyes, we see that the pigment rim is thicker than the wild type pigment rim (Figure 12 G-H). In order to understand the developmental events that lead to this adult phenotype, we examined the pupal eyes of *Pros-Gal4; UAS-Arm**. Until 30 hrsAPF, the eyes show a similar development to wild-type eyes, with the cone cells present apically over the photoreceptors (Figure 13 A-A'). At 32hrsAPF, all the cone cells express Esg (as observed with the *Esg-LacZ* transcriptional reporter) and Wg (as observed with anti-*Wg* antibody) (Figure 13 B-C). This indicates that all the cone cells express the peripheral molecular markers upon activation of Wg signaling. At 36hrs APF, similar to *GMR-Wg*, all the cone cells of the retina collapse to the level of photoreceptors (Figure 13 D-D'). But, at 42hrsAPF, we observe an interesting phenomenon. Apoptosis in the retina occurs in a broad peripheral region as opposed to the outermost row of ommatidia in a wild-type periphery (Figure 13 E-F). There is a qualitative increase in the number of ommatidia undergoing apoptosis at the periphery as compared to the apoptotic ommatidia at the wild type periphery, and yet most of these dying ommatidia are present at the peripheral regions of the eye (figure 13 E'). Although the cone cells in the main body of the retina have also collapsed, there is a very low level of sporadic apoptosis in the main body of these retinas (Figure 13 E''), unlike the apoptosis of the entire retina as seen in *GMR-Wg* eyes (Figure 13 G).

Based on these results, we infer the following – Activation of Wg signaling at high levels in the cone cells of the retina is sufficient for them to phenocopy the molecular responses of the peripheral cone cells. However, later developmental events show that the ommatidia of the retina display different responses depending on their position within the eye – ommatidia present at the peripheral regions (about 2-3 rows of outermost ommatidia) show cone cell collapse and apoptosis similar to the ommatidia of a *GMR-Wg* eye and the wild type peripheral ommatidia. We also infer that this wider region of ommatidial apoptosis probably contributes to the thickening of the pigment rim, as seen in the adult eyes of *Pros-Gal4; UAS-Arm** (Figure 12 G-H). Additionally, the ommatidia present in the main body of the *Pros-Gal4; UAS-Arm** eyes show collapse of all the cone cells, but there is no concerted ommatidial apoptosis. This cone cell collapse could be the reason for the occurrence of the degenerated, stunted ommatidia seen in the retina in the adult eye sections.

Based on the data presented above, we conclude that cone cell specific activation of Wg signaling is sufficient to elicit the appropriate molecular responses in the cone cells, and their subsequent collapse. However, this activation is not sufficient to cause the apoptosis and clearance of the ommatidia, as seen in the *GMR-Wg* pseudo periphery.

Importantly, we observe a wider region of death at the periphery and the formation of a thicker pigment rim in the *Pros-Gal4; UAS-Arm** eyes as compared to a wild type pigment rim. This suggests that we have generated two regions within the same *Pros-*

*Gal4; UAS-Arm** retinal field. One region is the broad zone at the periphery where ommatidia undergo apoptosis (similar to the wild type periphery and to the ommatidia of the *GMR-wg* eyes) to form a thicker pigment rim. The other region is the main body of the retina where cone cells have collapsed, there is low level sporadic apoptosis and adult eyes show degenerated photoreceptors and a disarrayed pigment lattice. We shall be using this peripheral zone of apoptosis in the *Pros-Gal4; UAS-Arm** eyes as an alternative pseudo periphery tool to further dissect out the roles of the individual cone cell gene expressions, and how they affect the observed phenotypes – namely the broad swathe of death at the periphery and the thickened pigment rim.

2.3 Role of the Wg induced molecular responses in the cone cells

To reiterate, the peripheral cone cells express Wg, Esg and Notum in response to the HC derived Wg signal. Notum was shown to function in restricting the diffusion of the Wg, thereby restricting ommatidial apoptosis to the outermost row (Lim and Tomlinson 2006). In the previous section, we showed that upon cell autonomous activation of Wg signaling, all the cone cells of the eye elicit similar molecular responses to the cone cells of the wild type periphery. Furthermore, we showed that this cone cell restricted Wg signaling activation produces two distinct phenotypes in the eye – degenerated, stunted ommatidia in the main body of the eye and a thicker pigment rim at the peripheral regions. We now wished to understand the role of the remaining molecular responses: What is the role of Wg and Esg in the formation of these phenotypes.

2.3.1 The role of Escargot in the cone cell response to Wg signaling activation

The next set of experiments we conducted was designed to investigate the effects of the loss of Esg from the cone cells on the peripheral death phenotype. As the *Pros-Gal4; UAS-Arm** eyes phenocopy the molecular responses of the peripheral cone cells and their subsequent collapse, as seen in the *GMR-Wg* pseudo periphery, we decided to test the effects of Esg removal from the cone cells in these *Pros-Gal4; UAS-Arm** eyes. For knockdown purposes, we decided to use *UAS-Esg-RNAi* (TRiP RNAi project), which has been reported to target both *esg* and *snail* transcripts, thus ensuring the removal of the majority of the Snail class transcription factors.

Effect of removal of Escargot in Pros-Gal4; UAS-Arm* eyes

When we analyzed the eyes of *Pros-Gal4; UAS-Arm*; UAS-Esg-RNAi* flies, we see that there is a dramatic restoration of the adult *Pros-Gal4; UAS-Arm** eye phenotype to a wild type appearance. The lenses look normal (figure 14 C'), and the eye size is comparable to wild-type (Figure 14 C). However, sections of these adult eyes show that the ommatidia are disorganized, in a fashion similar to *Pros-Gal4; UAS-Arm** eyes (figure 14 D''D'''). A majority of the photoreceptors still fail to extend the entire depth of the retina (as indicated by empty lattice spaces in Figure 14 C'''), and often the ommatidia lack the complete set of photoreceptors.

Interestingly, when we examined the peripheral sections of these *Pros-Gal4; UAS-Arm*; UAS-Esg-RNAi* eyes, we saw that instead of the thick pigment rim of *Pros-Gal4; UAS-Arm** eyes (Figure 14 E), there is a meshwork of empty (lacking

photoreceptors) ommatidial lattice adjacent to the HC (Figure 14 F). To better understand this adult phenotype, we decided to examine the pupal development of these *Pros-Gal4; UAS-Arm*; UAS-Esg-RNAi* eyes. The pupal development of the eyes of these animals shows that at 36hrs APF, the cone cells fail to collapse to the level of photoreceptors (Figure 15 A-A'). And at 42hrsAPF, we see that the cone cells are present normally at their apical positions (Figure 15 B), while the photoreceptors lying underneath them undergo apoptosis in a broad zone (figure 15 B') similar to the *Pros-Gal4; UAS-Arm** eyes. These results indicate that the removal of Esg from the cone cells of *Pros-Gal4; UAS-Arm** eyes prevents their collapse and apoptosis of the cone cells. However, this removal does not have any significant effect on either the degeneration of the photoreceptors in the main body of the eye, or on the apoptosis of the outer rows of photoreceptors. The empty lattice adjacent to the HC instead of the thick pigment rim suggests that the survival of these peripheral cone cells contributes to a partial rescue of the ommatidial structure, even if there are no photoreceptors within them.

The next idea was to test the effect of removing Esg in a wild type periphery. To do so, we examined the eyes of *Pros-Gal4; UAS-Esg RNAi* flies. The adult eye periphery of these flies shows the presence of small lenses adjacent to the head capsule (Figure 15 C), consistent with lenses secreted by incomplete peripheral ommatidia, but no photoreceptors underneath them in the deeper sections (Figure 15 C'). Pupal development analysis of these eyes shows Wg expression in the peripheral cone cells at 32hrsAPF (Figure 15 D), indicating that by manipulating Esg expression, we have

not altered the other cone cell responses. Similar to the *Pros-Gal4; UAS-Arm*; UAS-Esg-RNAi eyes*, the peripheral cone cells of *Pros-Gal4; UAS-Esg-RNAi* eyes do not collapse, and at 42hrsAPF, the outermost photoreceptors undergo apoptosis while the overlying cone cells survive (figure 15 E-E'). Taken together, these data indicate that removal of Esg prevents cone cell collapse, and causes a partial rescue of the ommatidial lattice structure. These results are also supported by the fact that Esg has been reported to be involved in regulation of cell adhesion molecules (Tanaka-Matakatsu, Uemura et al. 1996). However, removal of Esg from the cone cells has no effect on the apoptosis of the associated photoreceptors.

Effect of ectopic expression of Escargot in the cone cells of the entire eye

In the previous section, we have shown that removal of Esg prevents cone cell collapse and they do not undergo apoptosis subsequently. Next, we wished to examine the effect of ectopic Esg expression, thereby assessing the degree to which the Wg induced cone cell response occurs via activation of Esg expression. For ectopic expression of Esg, we utilized a *UAS-Esg* transgene, driven in the cone cells by *Pros-Gal4*. When we examined the eyes of *Pros-Gal4; UAS-Esg* flies, we saw that the eyes of the adult flies are smaller in size, with deformed lenses (Figure 16 B). The adult eye sections show that the main body of the retina contains only pigment cells, appearing similar to the *GMR-Wg* pseudo periphery (Figure 16 B'). However, deeper sections of the retina show that photoreceptors appear to have delaminated from their retinal positions and have fallen to the basal lamina (Figure 16 B''). To better

understand this phenotype, we checked the pupal development of the eye at different stages. Upon doing so, we find that there is a precocious collapse of the cone cells – as early as 30hrs APF (Figure 16 C-C'). This suggests that expression of Esg is sufficient to trigger the collapse earlier than the wild type periphery. This is what we would expect, since we have shown previously that removal of Esg prevents cone cell collapse. Subsequent stages show that the ommatidia start to clump together (Figure 16 D-D') but the apoptosis still appears to be restricted to the periphery (Figure 16 E-E'). Due to the severe tissue disruption, the analysis of later gene expressions and subsequent changes was not possible and therefore we cannot interpret these results in a satisfactory manner.

To conclude, the role of Esg expression in the peripheral cone cells appears to be the enabling of cone cell collapse, and destabilizing the ommatidial lattice structure. The removal of Esg expression from the cone cells has no significant effect on the degeneration and apoptosis of the photoreceptors. Also, the expression of Esg and the subsequent cone cell collapse appear to be independent of the other cone cell responses, namely Wg and Notum.

2.3.2 Role of cone cell derived Wingless in the cone cell response to Wingless from head capsule

In response to Wg diffusing in from the HC, the cone cells respond by expressing Wg, Esg and Notum. In the previous section, we showed that the Esg expression is required for the cone cell collapse. However, the removal of Esg failed to prevent the

peripheral photoreceptor apoptosis. This led us to question the role of the 2° Wg secreted by the cone cells. We hypothesized that this 2° Wg might act like a booster dose in addition to the Wg diffusing from the HC, thereby enabling only the outermost ommatidia to reach the high threshold response and undergo subsequent apoptosis.

Effect of removal of cone cell derived Wingless on the cone cell response to head capsule derived Wingless signaling

In order to specifically remove the 2° Wg produced by the cone cells without affecting downstream Wg signal transduction in the cone cells, we decided to use a *UAS-Wg-RNAi* transgene driven by *Pros-Gal4*. As the *UAS-Wg-RNAi* line has not been previously characterized, we tested the efficacy of *wg* knockdown by this transgene. To do so, we checked the expression of Wg protein in the cone cells of *Pros-Gal4; UAS-Arm*; UAS-Wg-RNAi* pupal retinas at 32hrsAPF. We know that all the cone cells of *Pros-Gal4; UAS-Arm** retina produce 2° Wg at 32hrs APF. If the *UAS-Wg-RNAi* transgene is indeed causing *wg* knockdown, we should see no Wg staining in the cone cells of the pupal retinas of *Pros-Gal4; UAS-Arm*; UAS-Wg-RNAi*. When we examine these pupal eye discs at 32hrsAPF, we see that Wg protein expression is extremely reduced in the cone cells, thus confirming that the *UAS-Wg-RNAi* transgene is functional (Figure 17 A-B). The HC Wg is still visible, indicating that the immunostaining is fine, and that Wg expression is indeed reduced in these eyes.

To test the effect of cone cell specific *wg* knockdown, we examined *Pros-Gal4; UAS-Wg-RNAi* flies. Surprisingly, the periphery of these eyes showed a normal pigment rim, with no evidence of surviving ommatidia close to the periphery. We infer that the peripheral ommatidia are experiencing high levels of Wg in a chronic manner, and since the RNAi is knocking down only the *wg* produced from the cone cells (2° Wg), the HC derived Wg is probably sufficient to induce apoptosis of the peripheral ommatidia. As a counter approach, we decided to knockdown *wg* in the cone cells in a *Pros-Gal4; UAS-Arm** eye using *UAS-Wg-RNAi*. In this scenario, all the cone cells of the retina are experiencing cell autonomous chronic Wg signaling similar to the periphery; but they are unable to produce the 2 ° Wg in response to this activation.

When we examined the adult eyes of the *Pros-Gal4; UAS-Arm*; UAS-Wg-RNAi* flies, we see that many lenses are restored, and externally the eye resembles a wild type eye, albeit the lens array appears slightly disorganized (Figure 17 D-D'). Upon sectioning these eyes, we see that the main body ommatidia are more wild type in appearance, frequently bear the full complement of photoreceptors (figure 17 D'') and a greater number of the photoreceptors extend the depth of the retina. Importantly, when we look at the periphery of these eyes, we find that there are a large number of surviving peripheral ommatidia instead of the thickened pigment rim previously described for *Pros-Gal4; UAS-Arm**eyes (figure 17 C'''). The thickness of the pigment rim appears similar to the wild type pigment rim(Figure 17 D'''), suggesting that we have inhibited the broader swathe of death by removing the 2° Wg signal. This set of results suggests that the 2° Wg produced by the cone cells in

response to the HC derived Wg is required for the degeneration of the ommatidia in *Pros-Gal4; UAS-Arm** eyes. It also appears to be one of the factors responsible for the broader zone of peripheral death in this genotype. These results will be further validated by pupal developmental analysis to assess the molecular and morphological events that lead to the inhibition of the broad zone of apoptosis. These data also suggest that in a wild type scenario, the cone cells produce 2° Wg as a booster dose, and they also produce Notum at the same time, thus serving to restrict the high level Wg signaling to the outermost row of ommatidia.

Effect of removal of both Esg and Wg from the cone cells at the periphery of the eye

In a *Pros-Gal4; UAS-Arm** eye, the observed responses to Wg diffusing in from HC are the expression of Wg and Esg in the cone cells followed by their collapse. Our previous results indicate that Esg is required for the collapse of the cone cells, and Wg is required for the ommatidial degeneration and the broad zone of peripheral apoptosis. Now we wished to find out what happens if we remove both these responses simultaneously from the cone cells of a *Pros-Gal4; UAS-Arm** eye. If these genes are responsible for the various phenotypes we have described above, then the removal of both these genes should restore a *Pros-Gal4; UAS-Arm** eye to a wild-type eye.

When we examined the eyes of *Pros-Gal4; UAS Arm*; UAS-Wg-RNAi; UAS-Esg-RNAi* flies, we saw that these eyes look almost wild-type externally. Sections through the adult eyes show a normal pigment cell lattice, with majority of the ommatidia

arranged normally (Figure 17 E). Occasionally we see ommatidia lacking a few photoreceptors; but most are wild-type in appearance and the rhabdomeres extend the depth of the retina. The periphery shows a few surviving ommatidia closer to the HC but looks almost wild-type (Figure 17 E'). It is difficult to assess the pigment rim area in these eyes as there is very little pigment in them. This is because the transgenes used in this experiment do not have eye pigmentation markers, thus making it hard to identify pigment cells. However, the appearance of ommatidia close to the HC is indicative of the fact that we have blocked the peripheral death (Figure 17 E').

The corresponding experiment in a WT periphery (*Pros-Gal4; UAS-Wg-RNAi; UAS-Esg-RNAi*) eye shows the survival of peripheral ommatidia adjacent to the HC (figure 17 F-F'). However the degree of inhibition of peripheral death is partial, which might be attributed to the fact that the RNAi lines do not accomplish a complete knockdown of the genes.

These results suggest that abolishing Wg induced cone cell responses is sufficient to block the ommatidial apoptosis to some extent, however the surviving ommatidia are not wild-type suggesting that there might be additional factors at work.

Effect of cumulative Wg signaling from the HC and from the cone cells

In a *Pros-Gal4; UAS-Arm** eye, we see that cone cells phenocopy the molecular responses of the peripheral cone cells, and all the cone cells collapse at 36hrsAPF. However, at 42hrsAPF, apoptosis occurs in a broad swathe of ommatidia of the outermost rows. In the previous sections, we have demonstrated that if we remove

one of the cone cell responses by removing Esg, we prevent the collapse and death of the cone cells in these *Pros-Gal4; UAS-Arm** eyes, however the underlying photoreceptors of these ommatidia still undergo apoptosis. We further showed that if we removed the Wg produced by the cone cells, we inhibit this broad region of death. But, this 2° Wg alone is insufficient to cause apoptosis, as seen in the main body of the *Pros-Gal4; UAS-Arm** eyes. Here, all the cone cells produce Wg and all of them collapse. However, these cone cells do not undergo the timed developmental apoptosis. This observation leads to the question – what is special about the outer region of the *Pros-Gal4; UAS-Arm** eye that makes the ommatidia susceptible to apoptosis? One possible explanation is that these outermost ommatidia might be experiencing additional signaling via the HC derived Wg; and that these two sources of Wg might be causing a synergistic effect to lead to ommatidial apoptosis.

If this hypothesis is true, then the addition of a tonic, low level expression of Wg to the main body of a *Pros-Gal4; UAS-Arm** eye should cause apoptosis and pigment rim like scarring in the middle of the eye. To test this hypothesis, we utilized the FLP/FRT recombinase system which causes site-specific recombination between two Flippase Recognition Target (FRT) in the presence of the recombinase enzyme, Flippase (Zhu and Sadowski 1995). To generate clones of Wg-producing cells in the middle of the eye, we use a transgenic construct consisting of *w+* cDNA (this is a mini-white gene, not the full *w+* cDNA, which results in a mild pigmentation in the eye) flanked by two FRT sites, placed downstream of a *Tub-α1* promoter. *wg-cDNA* is present further downstream of this "flp-out" cassette (Wehrli and Tomlinson 1998).

In the absence of Flippase, this transgenic construct will express *w+* gene under *Tub-α1* promoter control, but the stop codon prevents the *wg* cDNA from being transcribed. In the presence of flippase enzyme, the two FRT sites will recombine and lead to cis-acting excision of the "flp-out" cassette and the Tub-α1 promoter will be able to drive transcription of the *wg* cDNA. Depending on the cells that express flippase, we can generate clones of cells that are transcribing *wg* versus control cells that are transcribing the *w+* gene in the same eye. A major caveat which needs to be addressed at this point is: What levels of Wg will be generated by this transgene? If the amount of Wg produced is higher than the threshold required for the peripheral response, then we shall see apoptosis regardless of the experimental background. This transgene has been reported to generate clones of Wg that achieve the low level and intermediate level phenotypes i.e. balding and formation of DRO, but does not cause apoptosis (Tomlinson 2003), so it is suitable for our experiment.

Approach1 - Generation of *Tubα1>wg* clones by heat shock induction of flpase

In this approach, we used *flp* being driven under the control of a heat shock promoter (*hs-flp*) (Golic and Lindquist 1989). Under normal temperature (25°C), this promoter is inactive. At higher temperatures (30-37 °C), this promoter is active and causes the transcription of the flippase enzyme. To generate the clones, we subjected 1st instar larvae to a transient heat shock at higher temperatures (33°C), which leads to formation of mosaic tissue containing clones of cells that have excised the FRT recombination elements and clones of cells that still retain

it. In the control experiment [Tub-α1>*wg* clones generated in a wild type eye], we used *hs-flp* to generate clones in Tub-α1>*w+*>*wg* flies. In this scenario, the retinal cells are wild type. Only the cells that excise the cassette in response to *flp* induction will express low levels of Wg. Accordingly, Tub-α1>W*g* clones only show balding, corresponding to a low level Wg signaling response(Figure 18 B). Now we wished to examine what happens when these Tub-α1>*wg* clones are generated in the experimental *Pros-Gal4; UAS-Arm** eyes. Using this strategy, the first set of clones was induced at 24-48 hrs after egg laying (AEL). These clones were obtained at a very low frequency (possibly due to elimination of clones during earlier stages of eye development), but were large and showed smooth bald patches externally (Fig 18 A). Upon sectioning these eyes we saw that all the photoreceptors were degenerated and there was no lattice structure evident in the eye (figure 18 C). Unfortunately, this transgene carries a very weak *w+* eye color gene, so it was not possible to use pigmentation to demarcate clone boundaries. A possible explanation of this phenotype is that the low level of *Wg* generated in the large clones was sufficient to diffuse across the rest of the eye, therefore leading to degradation of ommatidia throughout the eye. We then decided to generate smaller clones to check if we could determine the extent of *Wg* diffusion and subsequent effects. Smaller clones were also generated at an extremely low frequency, and yet they showed a similar phenotype. Surprisingly, the eyes lacking the clones of the same genotype showed the same phenotype upon sectioning (figure 18 D). Because of the inexplicable variation of the *Pros-*

*Gal4; UAS-Arm** adult phenotype in this experimental set up, it is not possible for us to be confident about the effects of the low level *wg* clones. Therefore, due to these technical difficulties, we abandoned this approach.

Approach 2: Using *GMR-flp* to induce Tub-α1>*wg* throughout the retina

As an alternative approach, we decided to utilize a transgene where *flp* is expressed under the control of GMR enhancer element. When we examined the eyes of *GMR-flp;* Tub-α1>*w+>Wg* flies in a wild type background; the majority of the retinal cells excise the *w+* cDNA cassette and express Tub-α1>*Wg*. Accordingly these flies have bald eyes (Figure 19 A). When we cross these Tub-α1>*w+>Wg; GMR-flp* flies to *Pros-Gal4; UAS-Arm** flies, the resulting progeny flies have very small, "*GMR-Wg"* like eyes (Fig 19 C). Upon sectioning the adult eyes of this genotype, we see only a few patches of pigment, and there is no structure remaining in the eye (Figure 19 D). Owing to the fact that there is no pigment gene in any of these transgenes except *Pros-Gal4* (which bears a mini-white gene); it is very hard to determine the amount of pigment cells remaining in the experimental background. When we looked at the sections of *Pros-Gal4; UAS-Arm*;* Tub-α1>*w+>Wg* eyes (there is no w+ excision as there is no *GMR-flp*), the eyes are larger, with degenerated photoreceptor remnants and a disarrayed pigment cell lattice still present (Figure 19 E). This is similar to *Pros-Gal4; UAS-Arm** phenotype, thus indicating that the low level addition of Wg

causes the transformation of *Pros-Gal4; UAS-Arm** eye to *GMR-wg* like small eye.

The most striking difference between experimental and control eyes (lacking the *GMR-flp*) is the size of the eye – flies with the tonic dose of Wg added via *GMR-flp;* Tub-α1>*w+>Wg* added to the *Pros-Gal4; UAS-Arm** genotype have much smaller eyes. These results suggest that the addition of tonic levels of Wg to the retinal cells of *Pros-Gal4; UAS-Arm** is sufficient to cause the complete clearance of the degenerated ommatidia. It is difficult to assess the coalescing of the pigment cells owing to the lack of sufficient pigmentation in the transgenes used for this experiment. We still need to perform timed pupal development experiments to validate these results in a developmental context.

2.4 Summary of role of cone cells in Wg induced peripheral apoptosis

The data presented so far suggest that the cone cells appear to be the highest threshold responders in the moribund peripheral ommatidia, and their response to the Wg signal from HC leads to the downstream apoptotic events of the ommatidia. The cone cell response to HC derived Wg includes the expression of Esg, Wg and Notum at the periphery. Our experiments suggest that the Snail class proteins (Esg, Sna and Wor) are required and sufficient for the collapse of the cone cells. Wg is required for the subsequent clearance of these collapsed cone cells as well as for inducing the apoptosis of the associated photoreceptors. Previous studies in the lab showed that Notum, which is an α/β hydrolase that acts to restrict *Wg* diffusion, acts in the

peripheral cone cells to contain the range and potency of the *Wg* signal so that only the outermost row of ommatidia undergo apoptosis (Lim and Tomlinson 2006). Taken together, these results suggest that the cone cell response is the initial high threshold response of the ommatidia to the Wg signal. However, this primary response is not sufficient to cause apoptosis of the ommatidia at the periphery, thereby suggesting that the peripheral photoreceptors and pigment cells are also involved in responding to the HC derived Wg signal. These data indicate that the cells of the peripheral ommatidia respond to the high level Wg signal in a combinatorial manner, thus leading to their apoptosis and formation of the pigment rim.

Objective 3: Examining the role of photoreceptors in the Wg induced peripheral apoptosis

In the work presented so far, we have presented evidence to show that high levels of Wg lead to pigment rim formation at the periphery as a timed sequence of events. Furthermore, we showed that activation of Wg signaling at high levels in all the cells of the retina transforms the entire eye into a pigment rim like pseudo periphery. We then analyzed the role of the cone cells and showed that the activation of Wg signaling pathway in all the cone cells of the retina is insufficient to cause pigment rim like pseudo periphery transformation of the entire eye. Experimental evidence also suggests that there might be a synergistic interaction between the HC derived Wg and cone cell derived Wg (2^o Wg); and that this additive signaling might be the cause of photoreceptor apoptosis and subsequent clearance of the peripheral ommatidia. In this section, we will assess the role of the photoreceptors in the apoptotic response to high level Wg signaling.

Tools for photoreceptors specific gene expression

To understand the role of the photoreceptors, we searched for a Gal4 driver line expressed specifically in the photoreceptors to perform our gene expression manipulations. After testing for the fidelity of many published photoreceptor specific driver lines, using *UAS-GFP* as a reporter for their expression pattern, we found *Orthodenticle-Gal4* (*Otd-Gal4*) (Sprecher, Pichaud et al. 2007). This driver line shows strong expression restricted to the photoreceptor cells in the retina beginning in

the late 3rd larval instar stages and throughout pupal development (Fig 20). For all the photoreceptor specific analyses, we will be using this *Otd-Gal4* driver line.

3.1 Blocking Wg transduction in the photoreceptors

If the photoreceptors are a part of the Wg response cascade leading to apoptosis of the peripheral ommatidia, then, by removing Wg transduction in the photoreceptors we would expect prevention of the death of the ommatidia in the pseudo-periphery, as well as in the wild-type periphery. Using a similar strategy as with the cone cells, we used *UAS-Axn-GFP* (described in section 2.1) driven by *Otd-Gal4* to induce a cell autonomous blocking of the Wg signal transduction pathway specifically in the photoreceptors.

Blocking Wg transduction in photoreceptors in the GMR-Wg induced pseudo periphery

In a *GMR-Wg; Otd-Gal4; UAS-Axn-GFP* eye, all the cells of the retina are experiencing Wg signaling at high levels but the photoreceptors are unable to transduce the signal. Upon examining the adult eyes of this genotype, we see a dramatic rescue of the size of the eye (figure 21 C) as compared to a wild type eye (Figure 21 A). The eye is bald (Figure 21 C'), and only a few distorted lenses are seen on the surface (Figure 21 D). The adult eye sections show the presence of a large number of ommatidia (figure 21 D'); however, the photoreceptors are not wild-type in appearance. The ommatidia are frequently bearing incomplete sets of photoreceptors, and the rhabdomeres are distorted (Figure 21 D'). Most of the

photoreceptors are stunted and do not extend the full depth of the retina (Figure 21 D''). The pigment cell lattice is also abnormal (Figure 20 D'-D''). These results indicate that the apoptosis of photoreceptors in the pseudo periphery can be prevented to a large extent by blocking Wg transduction in the photoreceptors. However, the cone cells are probably not restored, as evidenced by the missing lenses on the eye surface. Pupal developmental analysis is required to understand the mechanism of this partial prevention of apoptosis in the pseudo periphery.

Blocking Wg transduction in the wild-type periphery

If by blocking Wg transduction in the photoreceptors, we can prevent the pseudo periphery death phenotype; then, under similar conditions, we can expect a similar effect at the wild-type periphery as well. In order to examine the effect of loss of Wg transduction in the peripheral ommatidia, we examined the periphery of *Otd-Gal4; UAS-Axn-GFP* eyes. At the periphery of these eyes, we see small, incomplete ommatidia present adjacent to the HC, but lacking the overlying lenses (figure 22 A-A'). To better understand what happens during the development of these eyes, we examined the pertinent pupal development stages. In a wild-type eye, the intermediate response to the HC derived Wg is the activation of the *homothorax* gene (Hth) in the central photoreceptors of the two outermost ommatidial rows in the dorsal half of the eye. These ommatidia will become the DRO (Wernet, Labhart et al. 2003). In the *Otd-Gal4; UAS-Axn-GFP* pupal eyes, at 27hrsAPF, there is no Hth in the central photoreceptors of the DRO (Figure 22 B), thus indicating that the molecular response

to Wg signaling is abolished in the photoreceptors. At 32hrsAPF, there is Wg expression in the peripheral cone cells (Figure 22 C), followed by their collapse at 36hrsAPF (figure 22 D), indicating that the cone cell responses are not altered. Interestingly, at 42hrsAPF, apoptosis at the periphery is extremely reduced, as compared to a wild type periphery (Figure 22 E). Occasionally we see a few dying cone cells but the outermost photoreceptors survive.

These results indicate that the photoreceptors do respond to the Wg induced "death signal" and that this response is required for the photoreceptor apoptosis and subsequent clearance to occur. Although these data do not clarify the distinction between the photoreceptors response to the HC derived Wg, versus their response to the 2° Wg secreted from cone cells; they strongly suggest that the Wg mediated peripheral death occurs via a combinatorial response from the cone cells and the photoreceptors.

3.2 Activating Wg signal transduction in the photoreceptors

In the previous section, we described the requirement of the photoreceptors' responsiveness to Wg signaling for the apoptotic cascade to occur. Also, data shown previously suggests that chronic exposure of high levels of Wg signaling to the photoreceptors causes their degeneration. If this is the case, then activation of Wg signaling in the photoreceptors should lead to their degeneration and apoptosis. To test this hypothesis, we utilized *UAS-Arm** (described in section 2.2) driven by *Otd-Gal4* to cause cell autonomous activation of Wg signaling in the photoreceptors.

Surprisingly, examination of *Otd-Gal4; UAS-Arm** eyes showed them to be entirely wild-type in appearance (Figure 23 A). The lenses and bristles on the surface of the eye are normal in appearance. Sections showed that apart from an occasional minor defect of improper separation of the photoreceptor rhabdomeres, the eye looked entirely wild-type (figure 23 B-C).

These results presented an unusual conundrum - blocking Wg transduction in the photoreceptors prevents photoreceptor apoptosis in the pseudo periphery, as well as in a wild-type periphery; but activating Wg transduction in the photoreceptors does not cause degeneration or apoptosis. To address these contradictory observations, we conducted a series of experiments to judge if this was a result of a technical anomaly or some other reasons.

Hypothesis 1 - The *Otd-Gal4* transgene might not be active at the correct pupal stage. To check this, we tested the expression of *UASdeGFP* (destabilized GFP) (Lieber, Kidd et al. 2011) with *Otd-Gal4* to get a more accurate temporal expression pattern, thus ensuring that this transgene is active during the pupal development phase we are interested in. This Gal4 line is expressed strongly in the photoreceptors throughout all stages of pupal eye development (Figure 24 A).

Hypothesis 2 - Our next idea was that the activation of Wg signaling might somehow be switching off the *Otd-Gal4* transgene in a regulatory negative feedback mechanism. To test this, we examined *Otd-Gal4; UAS-deGFP* expression in a *GMR-Wg* background. In this scenario, all the cells are experiencing high levels of Wg

transduction, so any effects on the *Otd-Gal4* transgene due to Wg signaling activation can be judged by the effects on the expression of the GFP reporter transgene. Again we found that *Otd-Gal4 line* is active throughout all stages of pupal eye development and is expressed only in the photoreceptors (figure 24 B-B').

Hypothesis 3 - The next idea was that the UAS transgene was being targeted by the feedback signal and maybe the Arm* transcription is being shut off. To test this, we used an activated Arm* transgene tagged with HA (*UAS-Arm*-HA*) (Zecca, Basler et al. 1996). When we stained the *Otd-Gal4; UAS-Arm*-HA* pupal eye discs with anti-HA antibody, all the photoreceptors showed the presence of the HA antigen (figure 24 C). This indicates that the transcription from the transgene is not affected.

Hypothesis 4 – Our next idea was that the Wg signaling pathway was not being activated because of a block in downstream Wg signaling, even though the Arm* transgene is being transcribed. To test if the constitutive Wg signaling pathway has actually been activated in these photoreceptors, we stained the *Otd-Gal4; UAS-Arm** pupal eye discs for Hth. All the central photoreceptors in the dorsal area stained positively for Hth, thus indicating that the Wg signaling cascade is indeed activate in these cells (figure 24 D).

Based on these data, we infer that the levels of Wg signaling in the eyes of these flies is sufficient to attain the intermediate dorsal rim phenotype, but not strong enough to cause photoreceptor apoptosis. We then tried doubling the copy number of either or both transgenes; however, we could never recover flies of the right genotype. We

infer that this might be a result of the Otd promoter being active during early developmental stages, thus potentially causing lethality upon driving higher levels of Wg signaling.

As an alternative approach, we used a tethered form of Wg (*UAS-Nrt-Wg*) to drive higher levels of Wg signaling in the photoreceptors in a cell autonomous manner. Upon examining *Otd-Gal4; UAS-Nrt-Wg* eyes, we saw that the adult eyes were smaller in size as compared to a wild type eye (Figure 24 F). The eye surface lacked lenses, but bristles were present. Sections through these adult eyes showed a complete absence of ommatidia (Figure 24G). The retina resembles *GMR-wg* pseudo periphery, however it is difficult to ascertain the status of the pigment cells owing to the lack of eye pigmentation markers in these transgenes. Furthermore, because the tethered Wg is expressed on the cell surface of the photoreceptors (Figure 24E), the adjacent cone cells and pigment cells can also respond to the Wg presented on the photoreceptor membranes. Thus, since the autonomy of the Wg signal is compromised, it is hard to interpret this result meaningfully.

Based on the results presented in this section, we conclude that while it is possible to activate Wg signaling specifically in the photoreceptors, and the intermediate level responses to Wg are observed; higher level responses and potential apoptosis could not be observed, probably because the survival of these flies is compromised.

3.3 What is the mechanism for Wg induced photoreceptor apoptosis?

In the previous section, we demonstrated that our experimental results regarding Wg signaling in the photoreceptors have led us to a conundrum – blocking Wg transduction in the photoreceptors prevents their apoptosis in the pseudo-periphery, and in the wild-type periphery. However, activating Wg transduction in the photoreceptors does not cause degeneration or apoptosis. This is contrary to the effects of Wg on the photoreceptors we observed in the *Pros-Gal4; UAS-Arm** flies. We hypothesized that the photoreceptors might be insensitive to Wg signaling because of the presence of overlying cone cells, but our previous data from experiments with *Pros-Gal4; UAS-Axn-GFP* and *Pros-Gal4; UAS-Esg-RNAi* indicate that even when the cone cell collapse is prevented, the underlying photoreceptors undergo apoptosis if they receive high enough levels of Wg signal.

Furthermore, our findings relating to cell autonomous activation of Wg signaling in the photoreceptors are contrary to the widely published claims that activation of Wg signaling in the photoreceptors causes their degeneration via apoptosis. We therefore decided to repeat the previously published experiments to assess if we could reproduce their results; and thus try and comprehend the data we generated with our *OtdGal4* experiments. Most of the published experiments have been conducted using the *LongGMR-Gal4* or *Elav (C155) Gal4* lines. However, the expression of these driver lines is not restricted to the photoreceptors, as demonstrated in the following experiments.

LongGMR-Gal4: *LongGMR-Gal4* is a driver line which is reported to be expressed specifically in the photoreceptors (Wernet, Labhart et al. 2003), but is actually expressed not only in the photoreceptors, but also in the cone cells and surrounding pigment cells (figure 25 A-A'). When we drive *UAS-Arm** with this driver line, we see a smaller eye as compared to a wild type eye (Figure 25 B). The eye looks rough with deformed lenses on the surface of the eye, the bristle array is also disrupted (Figure 25 B'). Sections through these eyes show that the photoreceptors are degenerated, and frequently do not extend the depth of the retina (figure 25 B''-B'''). The pigment cells are bunched together in a disorganized manner, as opposed to the regular hexagonal lattice (Figure 25 B''').

Elav(C155)-Gal4: *Elav (C155)-Gal4* line is the most widely used neuronal driver line that has been reported to show photoreceptor specific expression in the eye (Lin and Goodman 1994). The expression pattern of this line showed that along with the photoreceptors, there was strong expression in all the pigment cells (figure 26 A-A'). When we drive *UAS-Arm** with this driver line, we see a small, rough eye with fewer lenses and bristles (Figure 26 B). Sections through the adult eyes of *Elav(C155) Gal4; UAS-Arm** eyes show that the remaining lenses appear normal(Figure 26 B'), but all the photoreceptors have degenerated. The remaining pigment cell lattice appears to be empty and disorganized as compared to the regular hexagonal array seen in wild type eyes (Figure 26 B'').

The above experiments suggest that inducing photoreceptor apoptosis using Arm* is possible. However, because neither of these driver lines shows expression restricted to the photoreceptors, it is difficult to assess the photoreceptor specific response to Wg signaling based on these results.

APC: D-APC is reported to be a downstream member of the Wg signaling cascade. APC is a member of the Arm degradation complex, and acts to block Wg transduction in a dual manner – by binding and sequestering Arm in the cytoplasm, and by escorting the phosphorylated Arm to the proteasome for degradation (Kimelman and Xu 2006). Previously published work reports that D-APC mutant eyes show photoreceptor degeneration, and that this phenotype could be ameliorated by reducing levels of Wg signaling in these eyes (Ahmed, Hayashi et al. 1998).

Using the published allele D-APC^{Q8} (which is a mutation that leads to production of a truncated, non-functional version of the protein and is considered a null allele) (Ahmed, Hayashi et al. 1998), we examined the retinas of the D-APC^{Q8} homozygous mutant animals. We observed that these eyes look wild-type externally. Sections through the adult eyes show that underneath the normal array of lenses and bristles (Figure 27-A), there are no photoreceptors (Figure 27-A'). Few necrotic spots are indicative of the photoreceptor degeneration, but the surrounding pigment lattice array is perfectly wild-type (Figure 27-A').

When we examined the pupal development stages for the eyes of this genotype, we found that at 28hrsAPF, all the central photoreceptors of the dorsal area (and a few in

the ventral area) expressed Hth (fig 27 B). This suggests that there is activation of Wg signaling in all the photoreceptors of these ommatidia. At 32hrsAPF, the outermost row of ommatidia expressed Wg strongly in their cone cells, whereas cone cells in the main body of the eye showed relatively weaker Wg expression (Figure 27 C). This suggests that the cone cells throughout the retina might not be experiencing Wg signaling at high enough levels to elicit the full complement of the cone cell responses to Wg. This is further supported by the fact that the cone cells continue to lie apically in these retinas even at 42hrsAPF, while all the underlying photoreceptors undergo apoptosis (Figure 27 D-D'''). Taken together, these results suggest that Wg signal activation can lead to photoreceptor specific apoptosis. However, we have to take into consideration that in the case of D-APC^{Q8} homozygous mutants, the entire tissue is mutant i.e. the surrounding pigment cells as well as the cone cells are experiencing the same levels of Wg signaling as the photoreceptors, so they might be responsible for sending an additional "death signal" to the photoreceptors. Therefore this photoreceptor degeneration phenotype cannot be attributed to Wg signaling activation solely in the photoreceptors.

Although the experimental evidence provided so far suggests that Wg signaling causes photoreceptor apoptosis, there are a number of questions still unanswered: Although the levels of Wg signaling experienced by the D-APC homozygous mutant cone cells are not high enough to activate the high threshold cone cell response throughout the retina, the photoreceptors express Hth and undergo apoptosis. If the photoreceptors can undergo apoptosis in response to a lower threshold of Wg

activation, why don't we observe a similar 'death phenotype' with *Otd-Gal4;UAS-Arm*?

Another question – are the photoreceptors insensitive to Wg signaling? Do they require an additional signal from the associated cells of the ommatidia to respond to Wg? Or is there a block that prevents high level accumulation of Arm* in these photoreceptor cells? This last question is partially addressed by an experiment we conducted with *Elav(II)Gal4* driver line. This is an alternative neuronal driver line, which drives strong expression of target genes in two photoreceptors (R3/R4) during early larval development (figure 28 A). In the later larval stages and pupal stages, this driver line shows expression restricted to all the photoreceptors, although the levels of expression within the photoreceptors of an ommatidium are variable (Figure 28 B-B'). When we used this *Elav (II) Gal4* line to drive the expression of *UAS-Arm**, very few flies of the right genotype emerged. These flies have eyes that look externally wild-type, with normal bristles and lenses (figure 28 C). Sections through these eyes show that while the lenses and pigment cell lattice are all wild type in appearance (Figure 28 D), the underlying photoreceptors often show degeneration, with the number of photoreceptors within an ommatidium varying from 2-8 photoreceptors (figure 28 D'). This result suggests that strong chronic activation of Wg signaling specifically in the photoreceptors can cause differentiation and development defects in the photoreceptors. However, we need to analyze the pupal development stages to assess if the degenerated photoreceptors undergo the appropriate timed developmental apoptosis.

3.4 Summary of role of photoreceptors in Wg induced peripheral apoptosis

In this section, we have shown data that present a logical conundrum: While the prevention of Wg signal transduction in the photoreceptors prevents ommatidial apoptosis, activation of Wg transduction solely in the photoreceptors causes no death. Yet, there are multiple reports that claim photoreceptor apoptosis can be accomplished by activating Wg transduction. We have shown that in all of these published experiments; Wg signaling is activated not only in the photoreceptors, but also in the surrounding cells. We have also shown data indicating that strong chronic activation of Wg signaling in the photoreceptors might lead to their degeneration; however there is no clearance of the ommatidia to form the pigment rim. Taken together, these data suggest that photoreceptor apoptosis cannot be achieved by activating Wg transduction in the photoreceptors alone.

Figure panels

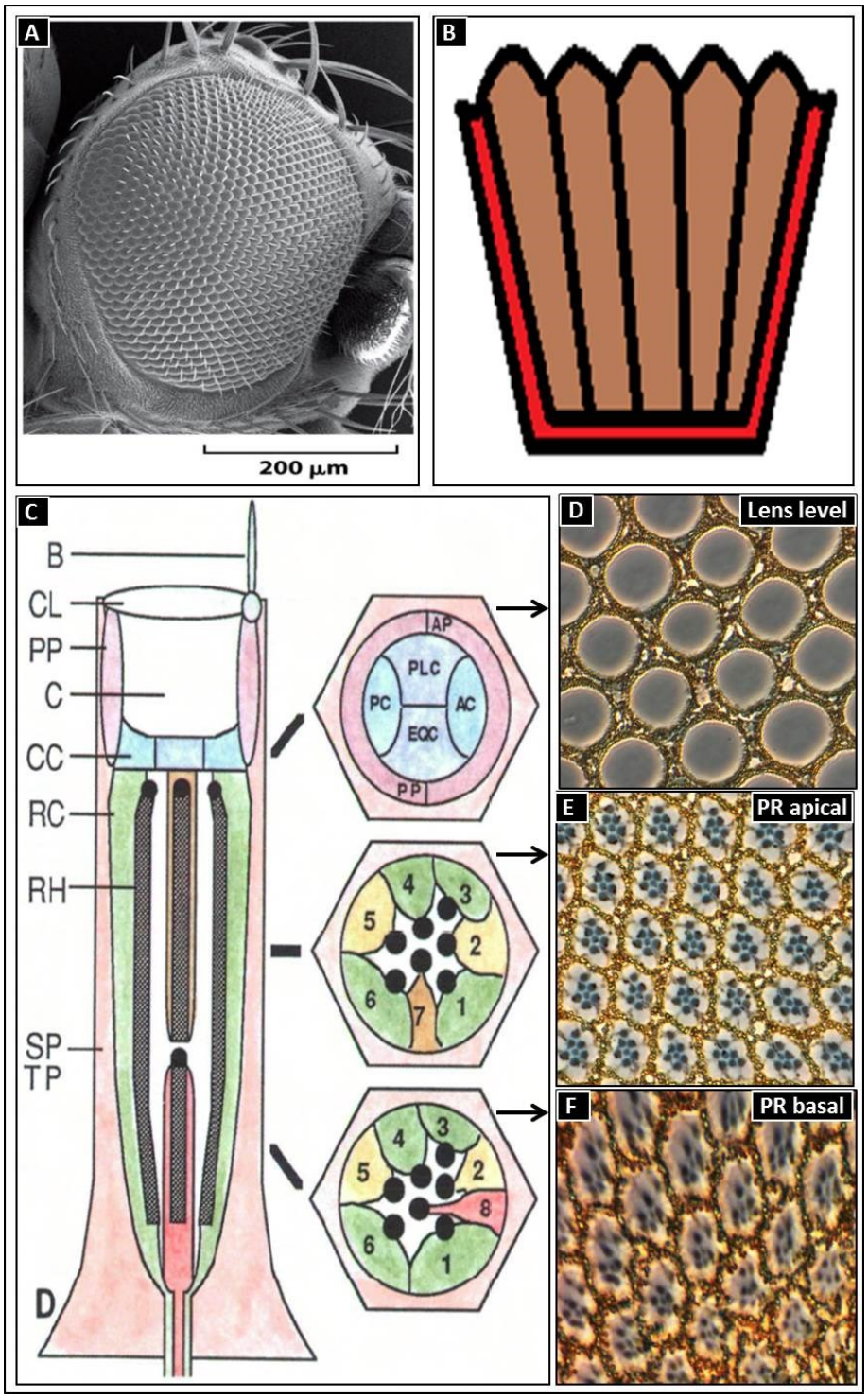

Figure 1: Structure of an ommatidium

A: Electron micrograph of an adult *Drosophila* eye

(Molecular Biology of the Cell, Garland Sciences 2008)

B: Cartoon figure depicting the arrangement of ommatidial units (in orange) in a niche made of pigment rim on the sides and Basal Pigment Layer at the base of the retina.

C: Cartoon representation of an ommatidium: B-Bristle, CL- Corneal lens, PP - 1°pigment cell, CC- Cone cells, RC – photoreceptor cells, RH- Rhabdomere, SP - 2°pigment cell, TP - 3°pigment cell

At the lens level, the four cone cells: Anterior(AC), Posterior(PC), Equatorial(EQC) and Polar(PLC) are present as a quartet.

The photoreceptors are present beneath the cone cells. In the apical photoreceptor region, the trapezoid if formed by photoreceptors R1 through R7.

In the basal photoreceptor regions, the trapezoid is formed by photoreceptors R1 through R6 and R8.

D: Section through apical region of the eye showing cross-section through lenses.

E: Section through the middle of the eye, showing the trapezoidal arrangement of photoreceptors in a hexagonal pigment cell lattice

F: Deeper sections through the adult eye showing the trapezoidal arrangement of photoreceptors in the more basal regions of the eye

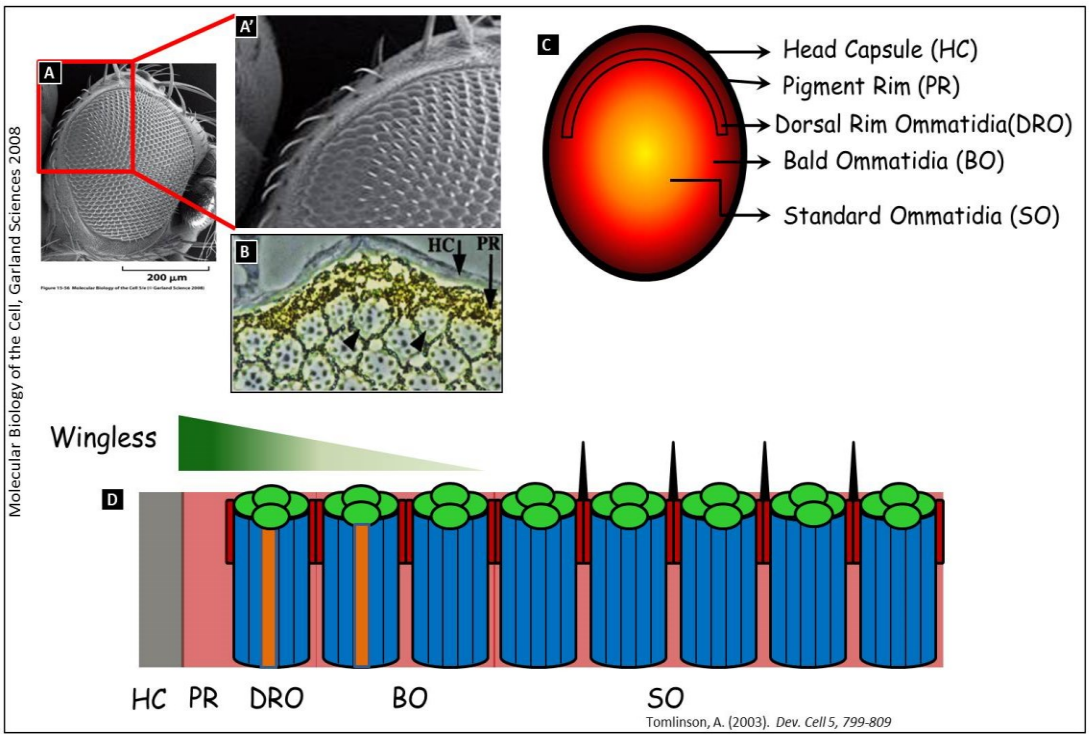

Figure 2: Peripheral specializations of the *Drosophila* eye as a result of a Wg gradient from the head capsule.

A: Immediately adjacent to the head capsule, outermost rows of ommatidia are bald (the bristles are missing-A')

B: Section through an adult eye showing the thick pigment rim (PR) adjacent to the head capsule(HC). The black arrowheads point to the big central rhabdomeres that distinguish the Dorsal Rim Ommatidia (DRO)

C: Cartoon representation of the specializations at the periphery of the eye

D: Cartoon depicting the Wg gradient that patterns the periphery of the eye

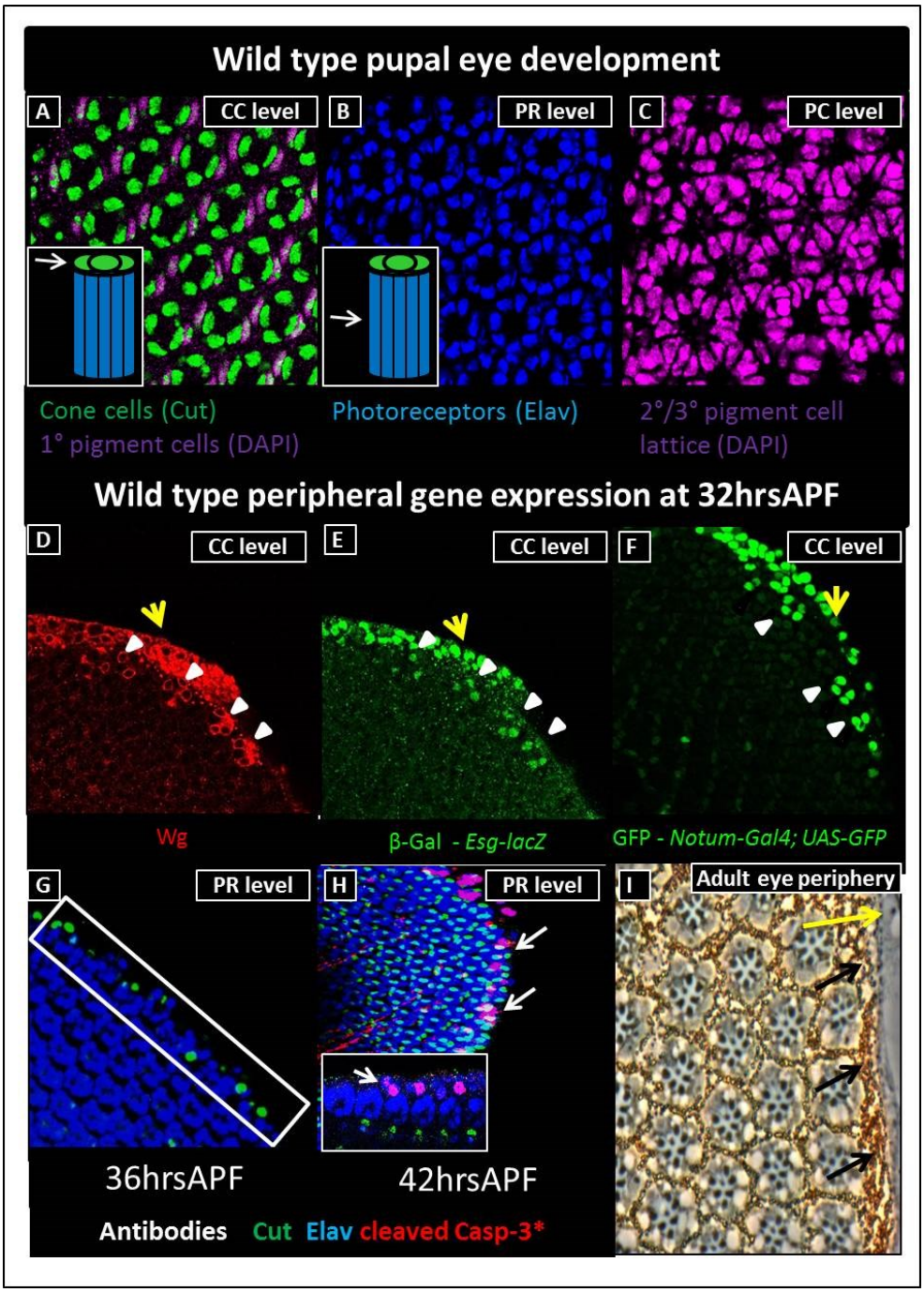
Wild type pupal eye development
A
CC level
B
PR level
C
PC level
Cone cells (Cut)
1° pigment cells (DAPI)
Photoreceptors (Elav)
2°/3° pigment cell
lattice (DAPI)
Wild type peripheral gene expression at 32hrsAPF
D
CC level
E
CC level
F
CC level
Wg
β-Gal - Esg-lacZ
GFP - Notum-Gal4; UAS-GFP
G
PR level
H
PR level
I
Adult eye periphery
36hrsAPF
42hrsAPF
Antibodies Cut Elav cleaved Casp-3*

Figure 3: Wild type pupal eye development at the periphery

A: Cone cells and 1°Pigment cells are present in the more apical planes, referred to as the 'CC level'. Cartoon depicts the focal plane.

B: Photoreceptors are present beneath the cone cells, at the 'PR level'. Cartoon depicts the focal plane.

C: 2°/3° pigment cell lattice at the base of the retina.

D-H: Wild type pupal development events at the periphery of the eye

D-F: Expression of Wg, Esg and Notum in the cone cells of the peripheral ommatidia at 32hrsAPF(marked by white arrowheads). Yellow arrow indicates the HC.)

G: At 36hrsAPF, the peripheral cone cells lose their apical position and collapse to the PR level (marked by the white box).

H: At 42hrsAPF, the outermost ommatidia undergo apoptosis, as evidenced by presence of caspase-3 staining. Inset shows that only the outermost ommatidia undergo apoptosis.

I: Peripheral section through adult eye. Yellow arrow marks the head capsule, black arrows point to the adjacent pigment rim.

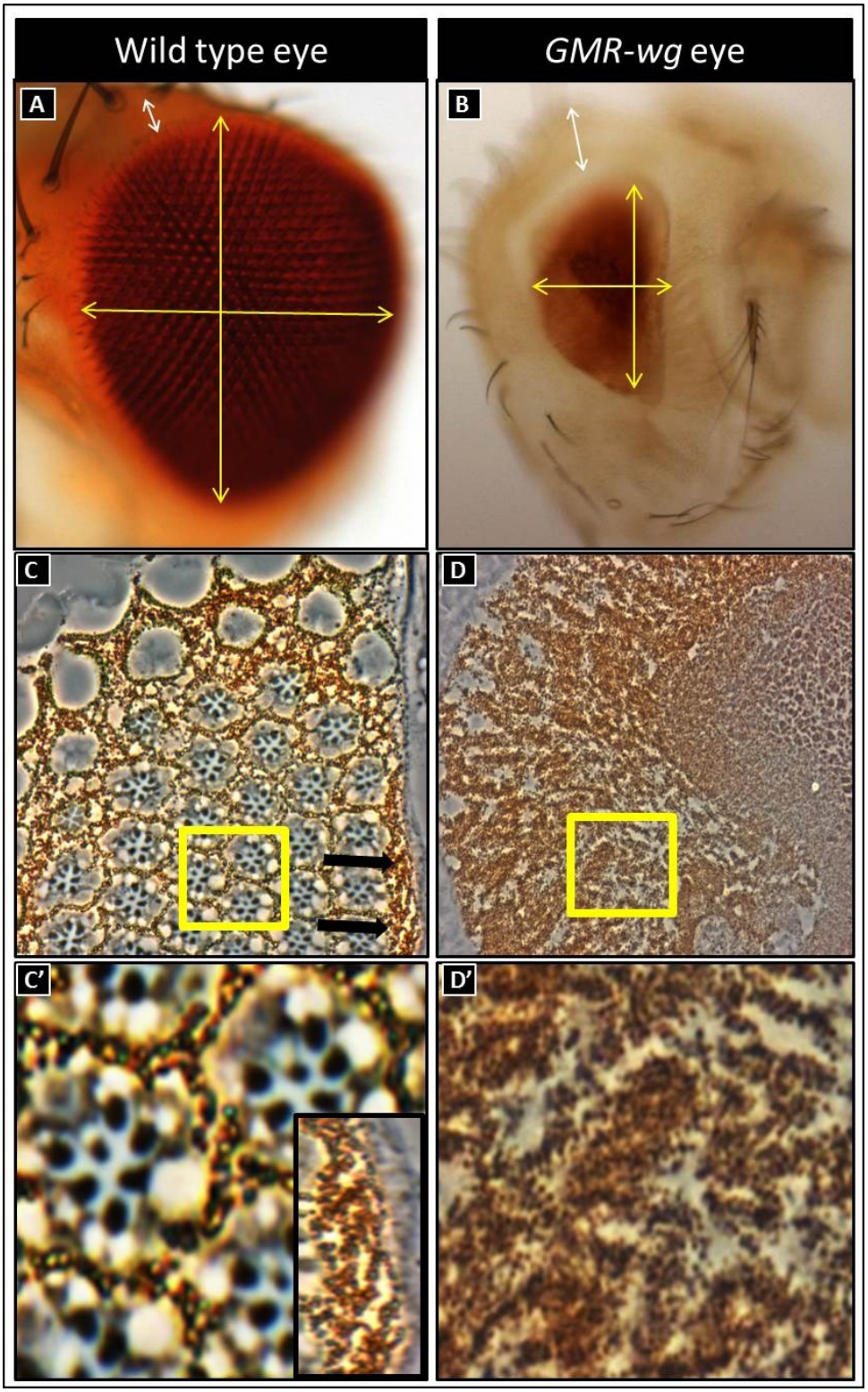
Wild type eye
GMR-wg eye
A
B
C
D
C'
D'

Figure 4: *GMR-wg* transforms the entire eye to a pigment rim – like 'pseudo periphery'

A: Whole mount of a wild type eye

B: Whole mount of a *GMR-wg* eye. The yellow arrows indicate the reduction in size of the eye field, the white arrows indicate the expansion of the head capsule.

C: Section through a wild type eye, showing the normal ommatidial array.

D: Section through *GMR-wg* eye shows that the eye contains only pigment cells.

C'-D': Magnified images of the yellow boxed areas. The *GMR-wg* eye tissue lacks any ommatidial structures, and resembles the pigment rim tissue (shown in inset in C').

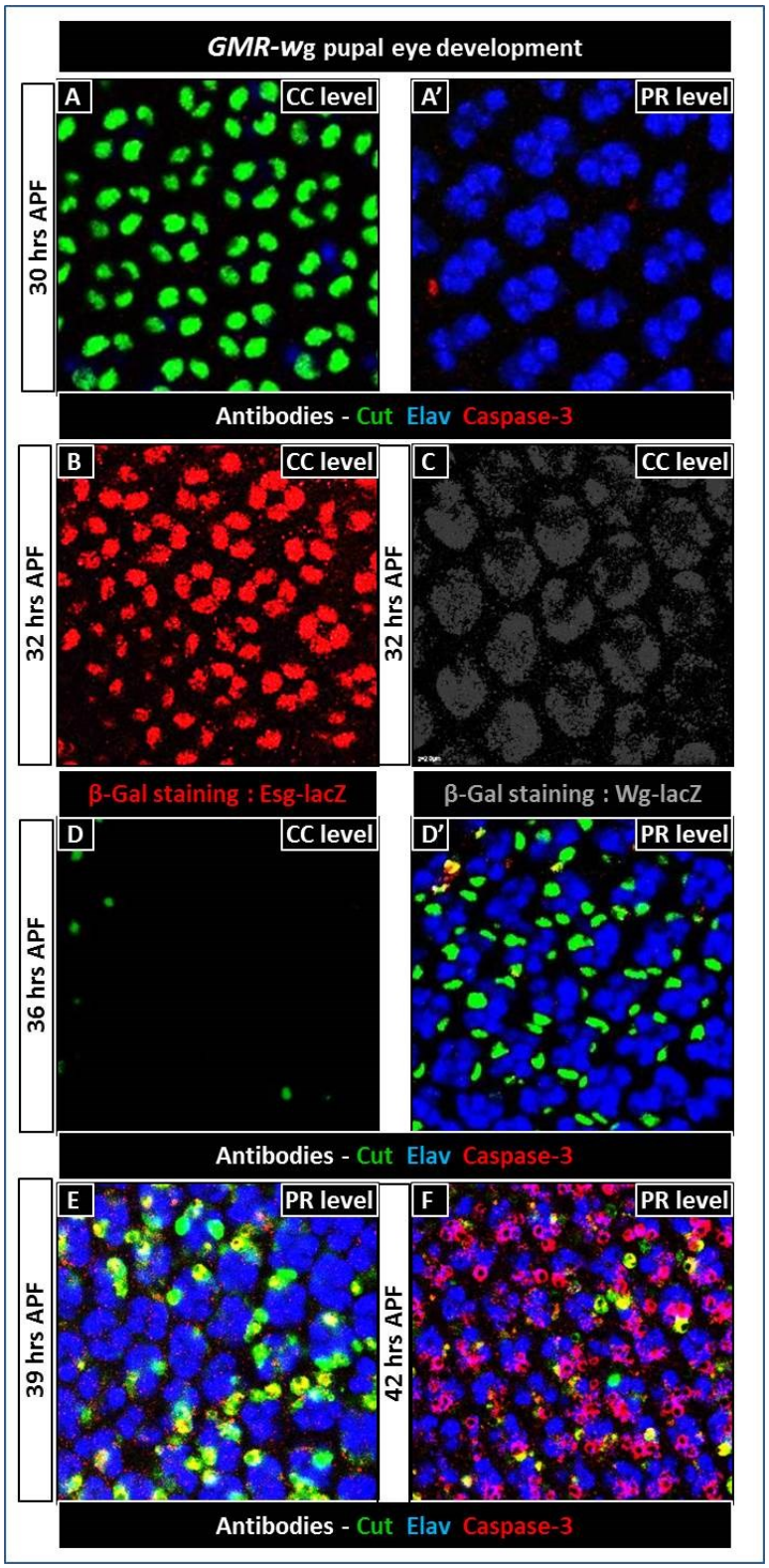
GMR-wg pupal eye development
A
CC level
A'
PR level
30 hrs APF
Antibodies - Cut Elav Caspase-3
B
CC level
C
CC level
32 hrs APF
32 hrs APF
β-Gal staining : Esg-lacZ
β-Gal staining : Wg-lacZ
D
CC level
D'
PR level
36 hrs APF
Antibodies - Cut Elav Caspase-3
E
PR level
F
PR level
39 hrs APF
42 hrs APF
Antibodies - Cut Elav Caspase-3

Figure 5: Pupal development of *GMR-wg* retina phenocopies peripheral development events of a wild type eye.

A, A': Up to 30hrsAPF, the eye shows wild type development, with cone cells present apically and photoreceptors beneath them.

B, C: At 32hrs APF, all the cone cells of the eye express Esg (B)and Wg (C), as evidenced by the transcriptional reporter lines *Esg-LacZ* and *Wg-LacZ*. (Images courtesy Dr. H. Patel)

D, D': At 36hrs APF, all the cone cells collapse to PR level.

E: At 39hrsAPF, cone cells undergo apoptosis, as evidenced by the co-staining of Cut antibody with caspase antibody.

F: AT 42hrsAPF, the entire eye is full of apoptotic cells.

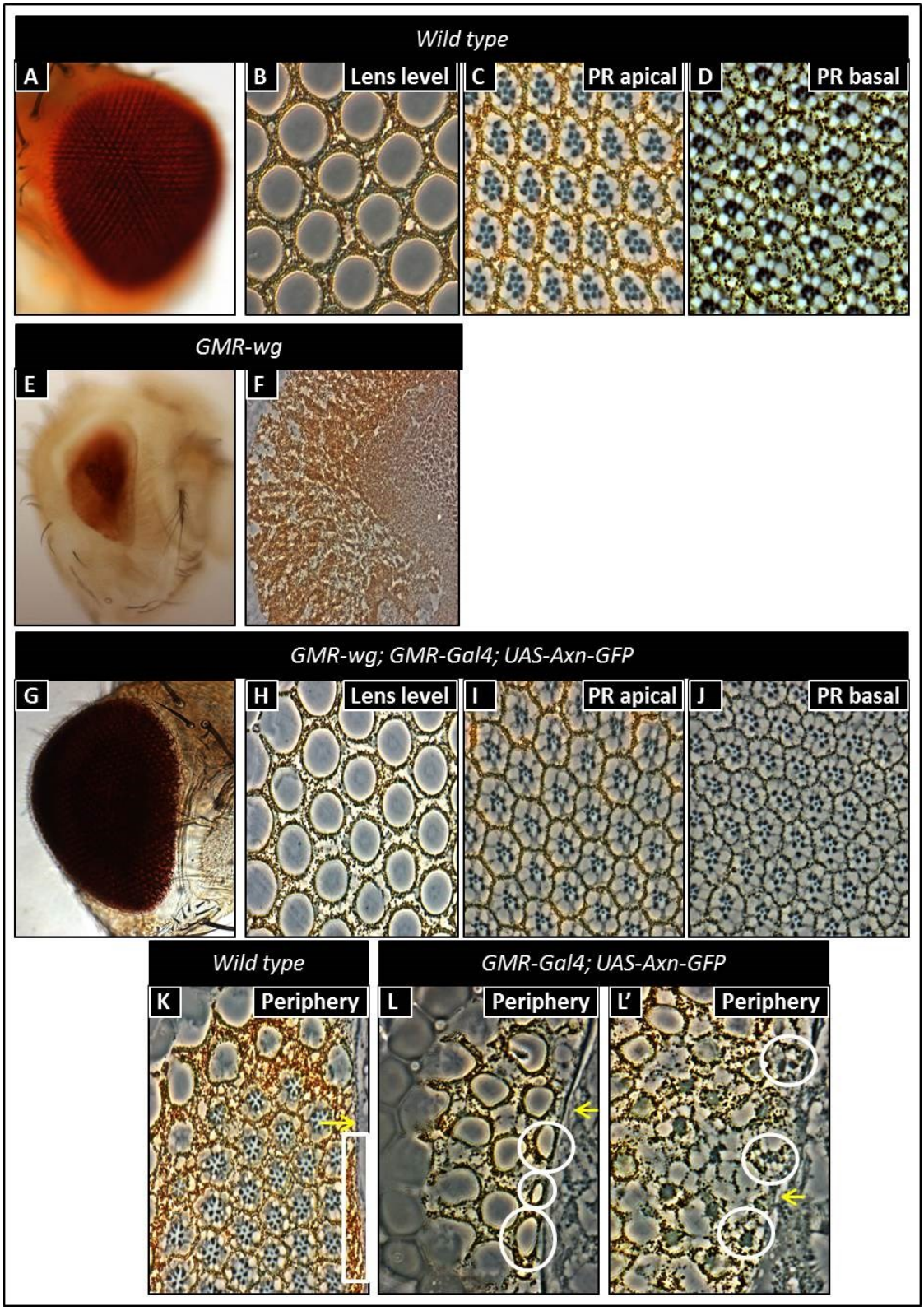
Wild type
A
B
Lens level
C
PR apical
D
PR basal
GMR-wg
E
F
GMR-wg; GMR-Gal4; UAS-Axn-GFP
G
H
Lens level
I
PR apical
J
PR basal
Wild type
K
Periphery
GMR-Gal4; UAS-Axn-GFP
L
Periphery
L'
Periphery

Figure 6: Blocking Wg transduction in the ommatidia is sufficient to prevent their apoptosis

A-D: Wild type eye, and the sections show normal array of ommatidia at the three focal planes – Lens level, PR apical and PR basal.

E-F: *GMR-wg* eye, sections show pseudo periphery tissue full of pigment cells.

G-I: Blocking Wg transduction in a *GMR-wg* eye using *GMR-Gal4; UAS-Axn-GFP* is sufficient to restore the retina to a wild type appearance, as evidenced by the images taken at the three planes.

K: Wild type periphery. HC marked by yellow arrow and pigment rim shown in boxed area.

L-L': Periphery of *GMR-Gal4; UAS-Axn-GFP* eyes. White circles mark the small lenses adjacent to the HC (L), deeper sections show the presence of incomplete peripheral ommatidia, and reduced PR region (L'). HC is marked by yellow arrows.

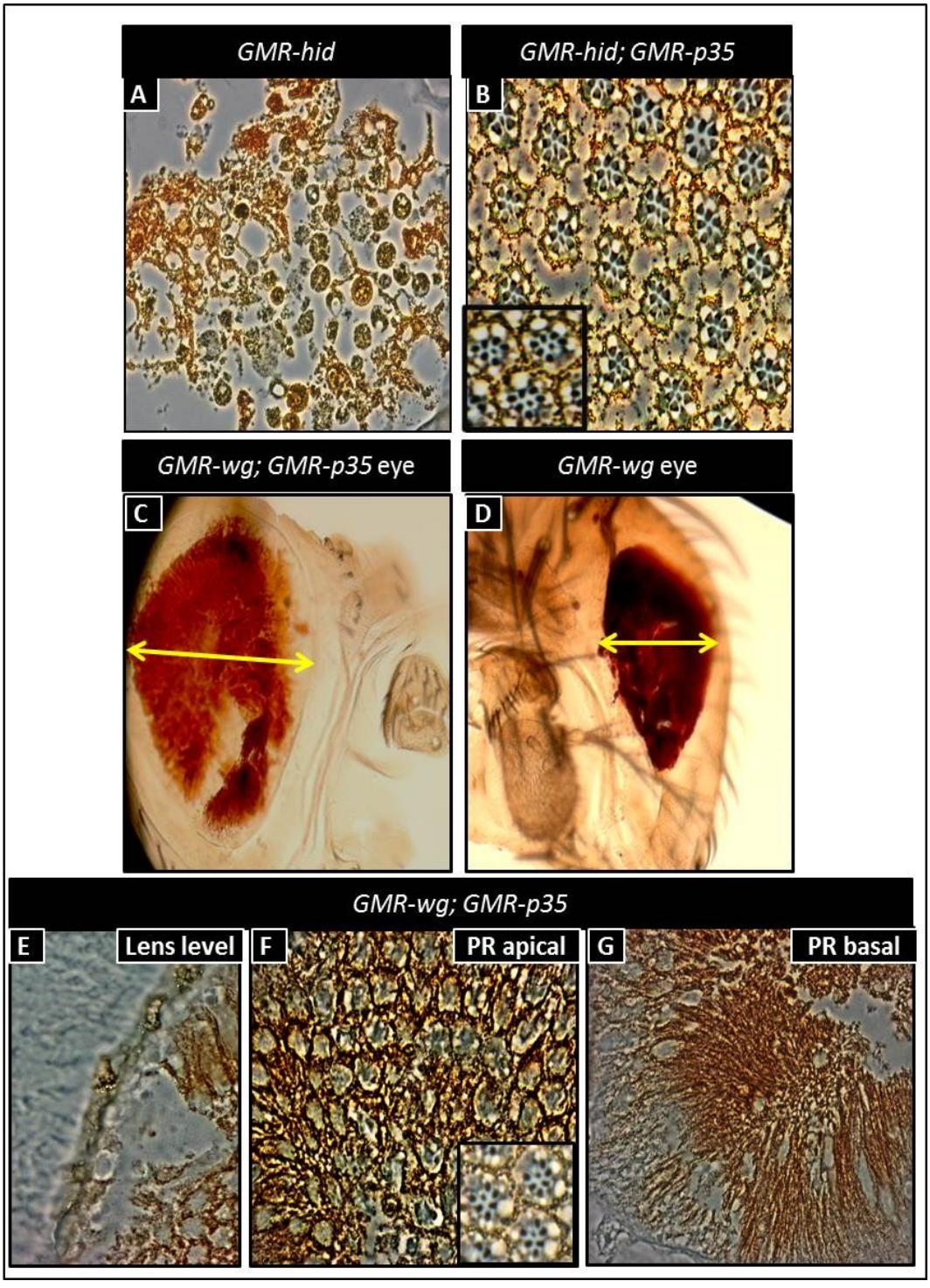
GMR-hid
A
GMR-hid; GMR-p35
B
GMR-wg; GMR-p35 eye
C
GMR-wg eye
D
GMR-wg; GMR-p35
E
Lens level
F
PR apical
G
PR basal

Figure 7: Blocking apoptosis is insufficient to restore *GMR-wg* eye to a wild type eye.

A: Section through a *GMR-hid* eye shows only clumps of pigment present in the eye.

B: Section through *GMR-hid; GMR-p35* eye shows complete restoration of the eye to a wild type eye(inset). There are extra inter-ommatidial cells present.

C: *GMR-wg; GMR-p35* eye shows a restoration of eye size to wild type proportions, as compared to a *GMR-wg* eye(D).

E-G: Sections through *GMR-wg; GMR-p35* eye. The eye is flat and bald on the outside, indicating lenses are not rescued (E). Many ommatidia seen at PR apical level(F), but they do not extend the depth of the retina. PR basal sections show pigment rim like appearance (G).

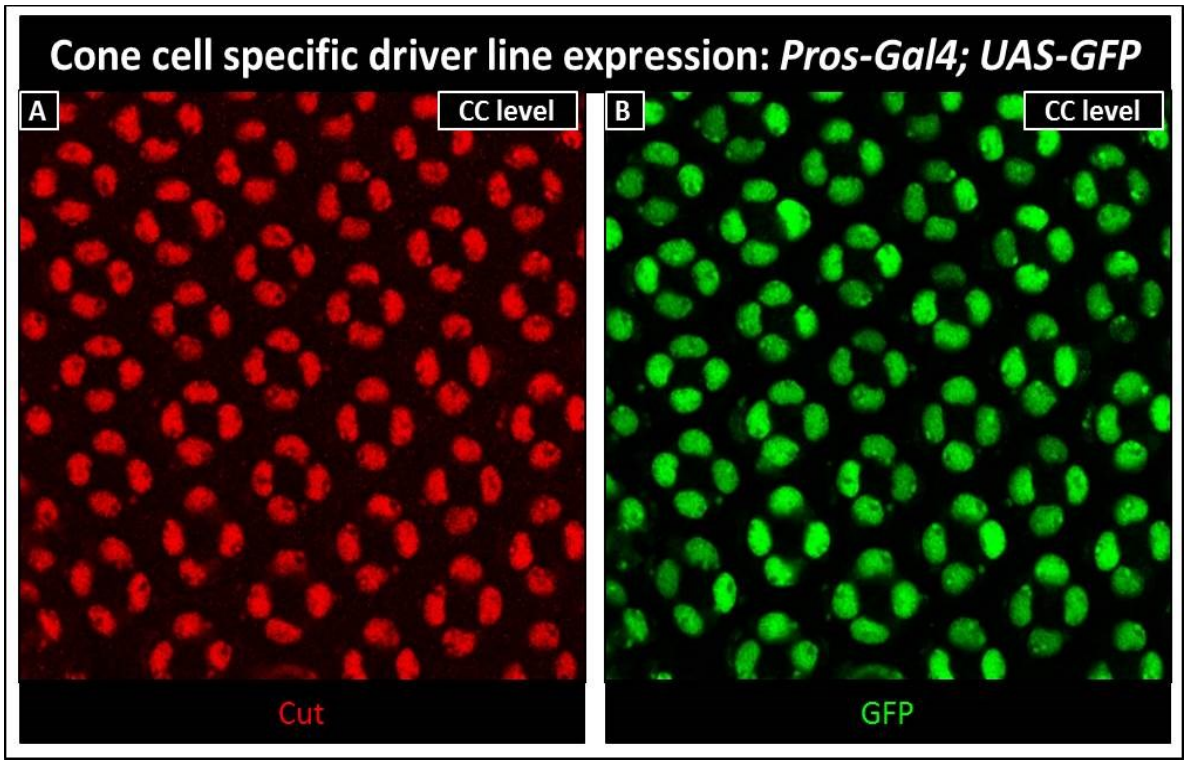

Figure 8: Expression of cone cell specific driver line: *Pros-Gal4.*

A: Cone cells marked by Cut antibody.

B: *UAS-GFP* driven by *Pros-Gal4* shows expression only in the cone cells at this focal plane, as evidenced by GFP antibody.

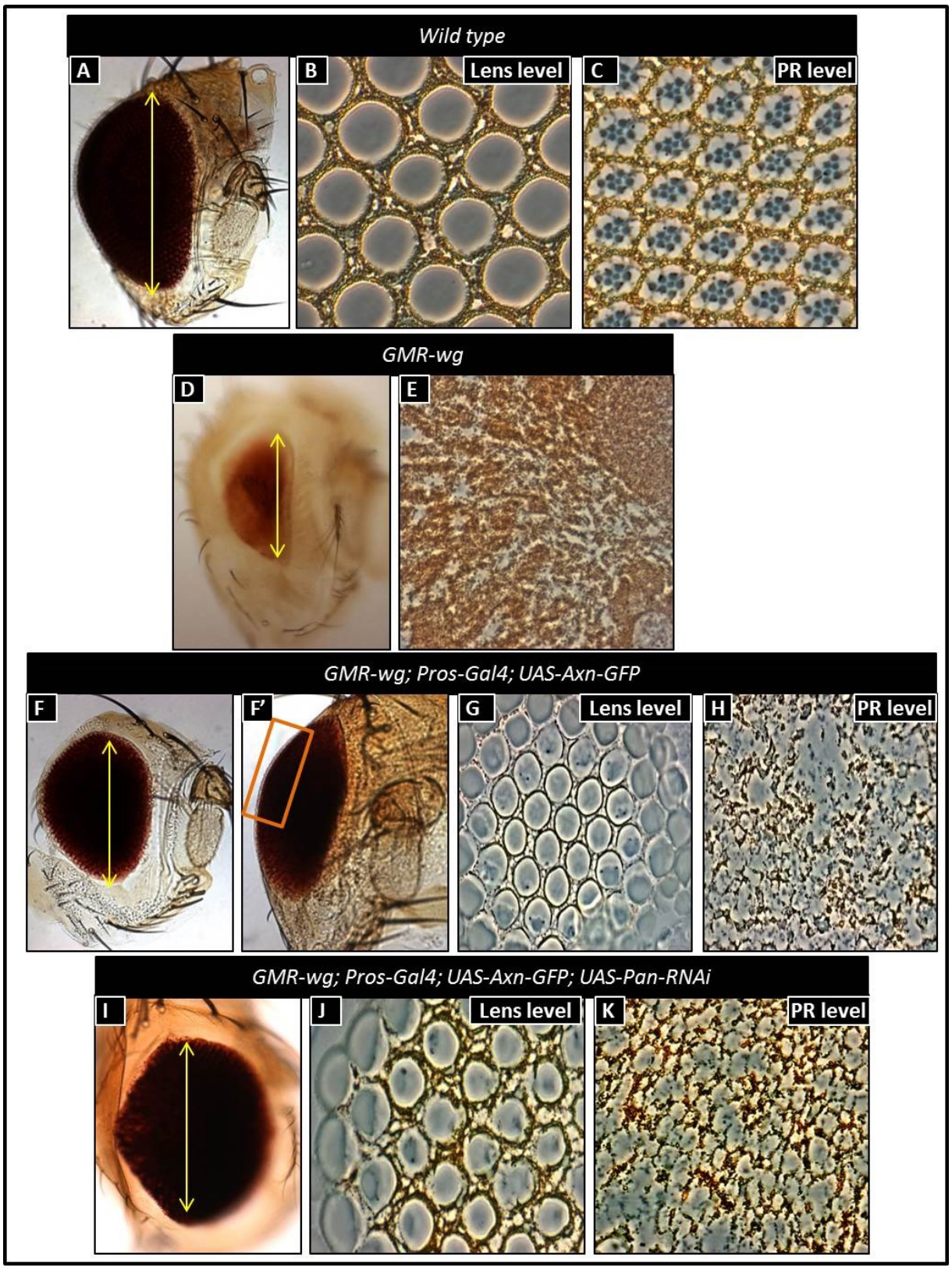
Wild type
A
B
Lens level
C
PR level
GMR-wg
D
E
GMR-wg; Pros-Gal4; UAS-Axn-GFP
F
F'
G
Lens level
H
PR level
GMR-wg; Pros-Gal4; UAS-Axn-GFP; UAS-Pan-RNAi
I
J
Lens level
K
PR level

Figure 9: Blocking Wg transduction in the cone cells only leads to partial rescue of *GMR-wg* eye phenotype

A-C: Wild type eye, and the sections show normal array of ommatidia at the lens level and at the PR level.

D-E: *GMR-wg* eye, sections show pseudo periphery tissue full of pigment cells.

F-H: *GMR-wg; Pros-Gal4; UAS-Axn-GFP* eyes. There is a partial rescue of size, but the eye still lacks bristles(orange box, F'). Sections through lens level (G) shows wild type array of lenses, but at the PR level (H) we see complete absence of ommatidia.

I-K: Similar results were obtained with *GMR-wg; Pros-Gal4; UAS-Axn-GFP; UAS-Pan-RNAi* eyes.

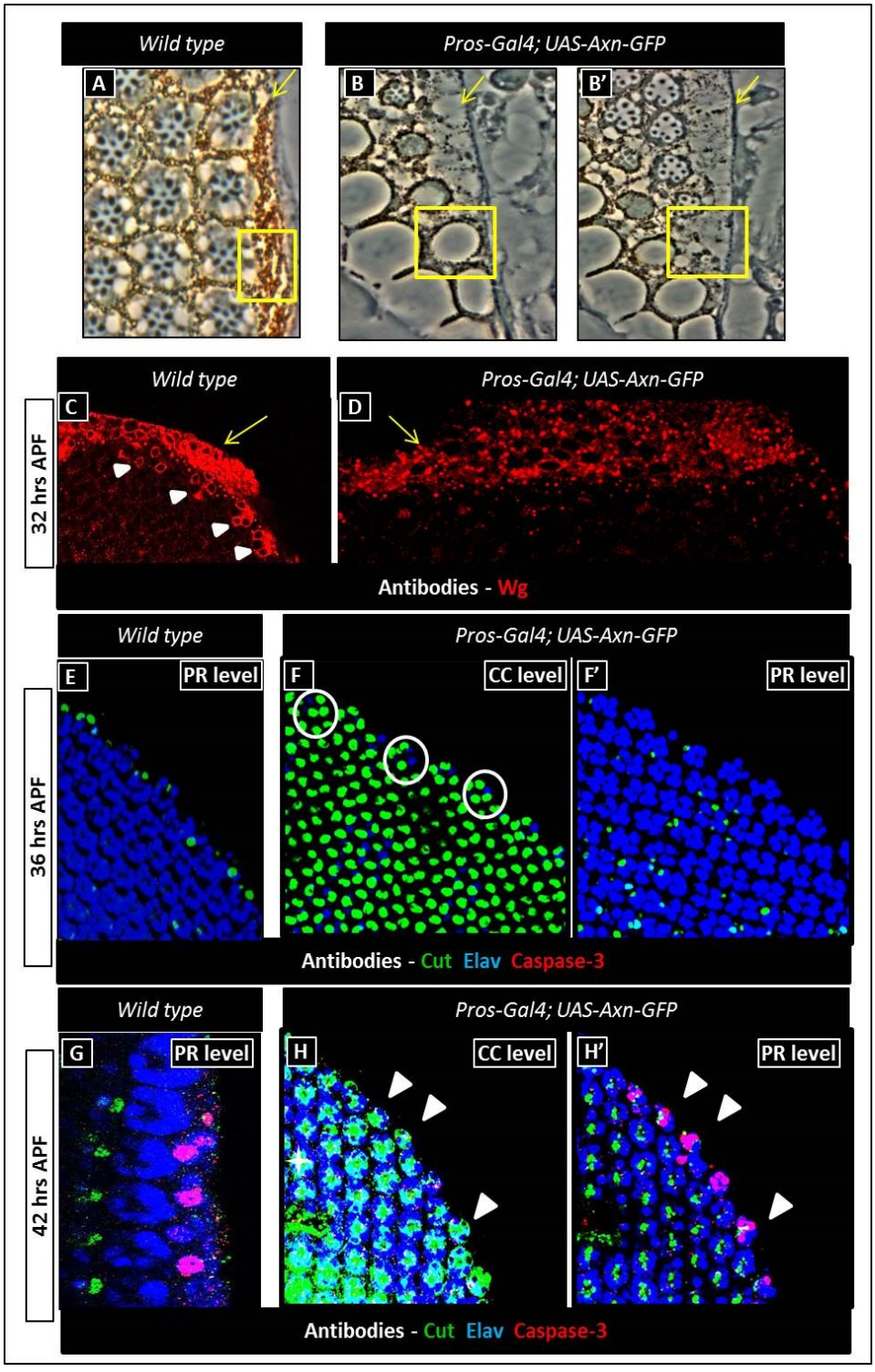
Wild type
Pros-Gal4; UAS-Axn-GFP
A
B
B'
Wild type
Pros-Gal4; UAS-Axn-GFP
C
D
32 hrs APF
Antibodies - Wg
Wild type
Pros-Gal4; UAS-Axn-GFP
E
PR level
F
CC level
F'
PR level
36 hrs APF
Antibodies - Cut Elav Caspase-3
Wild type
Pros-Gal4; UAS-Axn-GFP
G
PR level
H
CC level
H'
PR level
42 hrs APF
Antibodies - Cut Elav Caspase-3

Figure 10: Blocking Wg transduction in the cone cells and its effect on peripheral events

A: Wild type eye periphery showing pigment rim in boxed area. HC marked by yellow arrow.

B-B': Peripheral sections of *Pros-Gal4; UAS-Axn-GFP* eyes show presence of small lenses adjacent to the HC, but no photoreceptors underneath them. HC marked by yellow arrow.

C-D: Wg expression in the peripheral cone cells at 32hrsAPF is abolished in *Pros-Gal4; UAS-Axn-GFP* eyes (D). The HC Wg expression is unaffected (marked by yellow arrow).

E-F': AT 36hrsAPF, the peripheral cone cells of *Pros-Gal4; UAS-Axn-GFP* eyes(marked in white circles-F) fail to collapse to PR level; unlike their wild type counterparts(E).

G-H': At 42hrsAPF, the cone cells remain apically at the CC level (H) while the underlying photoreceptors undergo apoptosis(H'), similar to the wild type periphery (G).

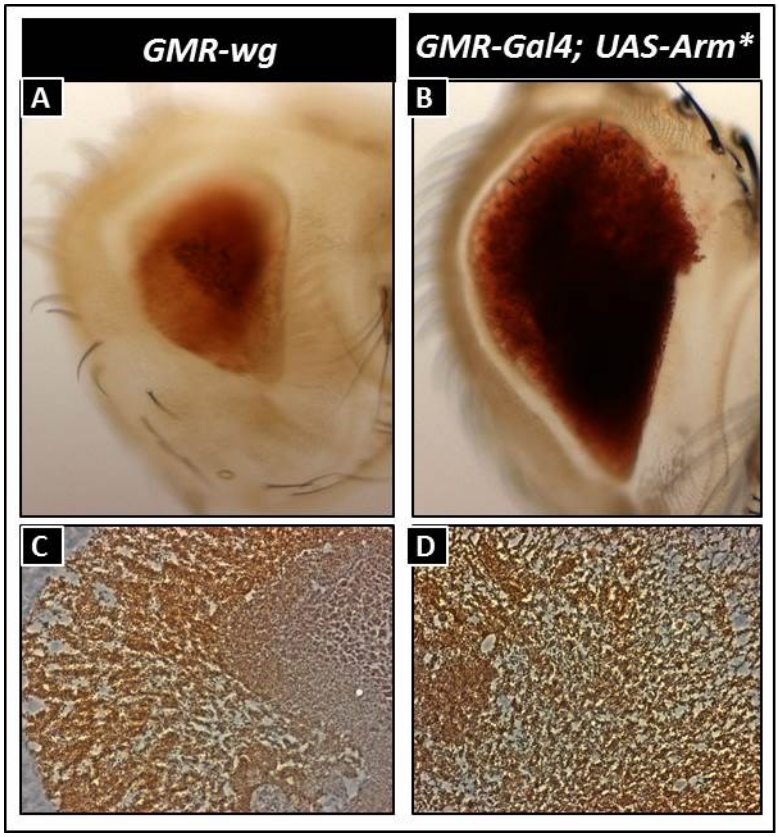

Figure 11: *GMR-Gal4; UAS-Arm** reproduces the *GMR-wg* pseudo periphery phenotype.

A, B: The adult *GMR-Gal4; UAS-Arm**eye (B) is slightly larger in size than a *GMR-wg* eye(A).

C-D: Sections through the *GMR-Gal4; UAS-Arm**eye(D) show a similar pseudo periphery like phenotype, as seen with *GMR-wg* eye sections(C).

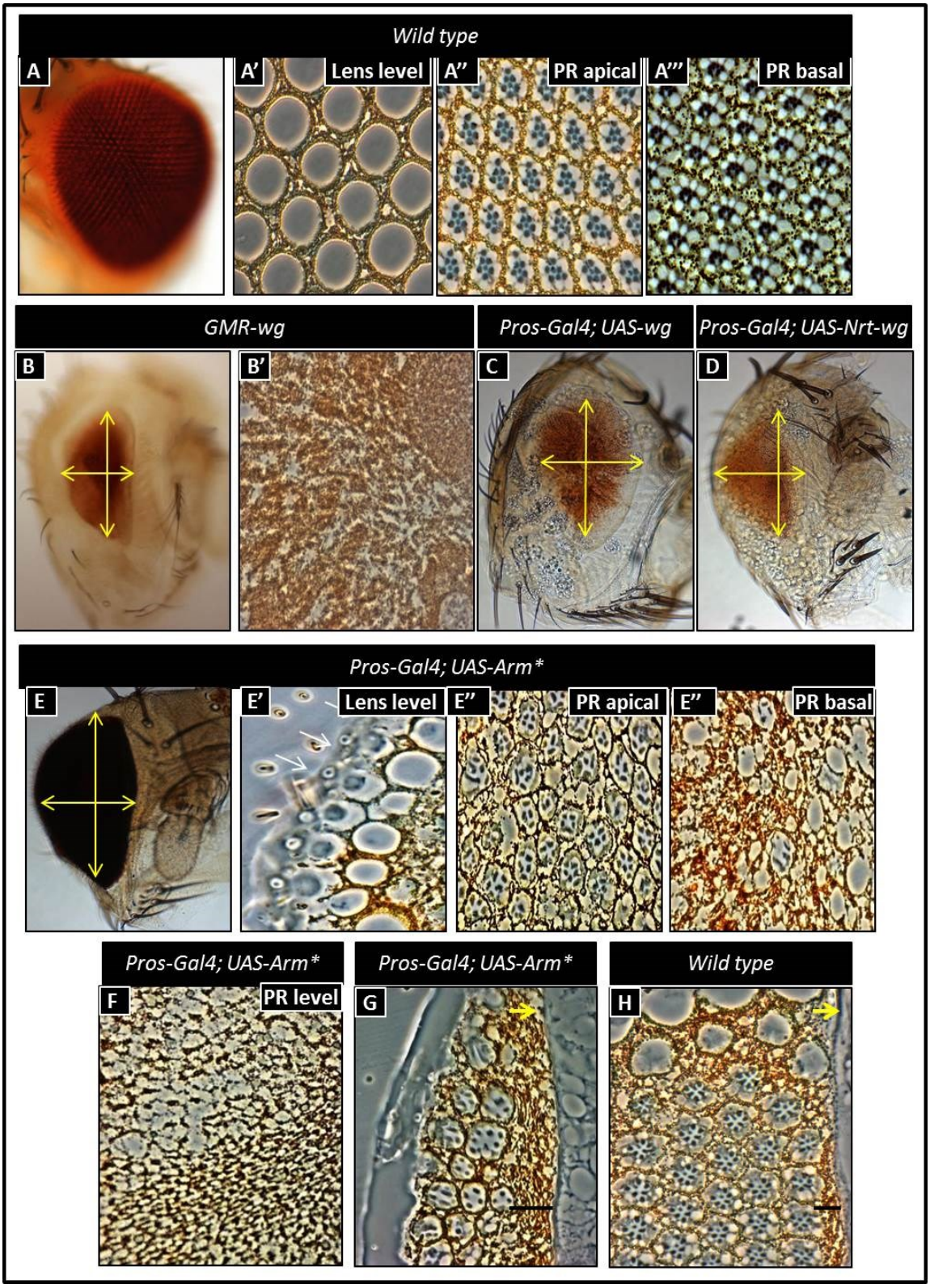
Wild type
A
A'
Lens level
A''
PR apical
A'''
PR basal
GMR-wg
Pros-Gal4; UAS-wg
Pros-Gal4; UAS-Nrt-wg
B
B'
C
D
Pros-Gal4; UAS-Arm*
E
E'
Lens level
E''
PR apical
E''
PR basal
Pros-Gal4; UAS-Arm*
F
PR level
Pros-Gal4; UAS-Arm*
G
Wild type
H

Figure 12: Effect of activating Wg transduction cell autonomously in all the cone cells of the eye

A-A’’’: Wild type eye and sections at the different focal planes.

B-B’: *GMR-wg* adult eye and section.

C-D: *Pros-Gal4; UAS-wg*(C) *and Pros-Gal4;UAS-Nrt-wg*(D) eyes show a *GMR-wg* like small eye phenotype.

E-E’’’: *Pros-Gal4; UAS-Arm** eye and sections. The adult eye is smaller than Wild type ey€. The lenses are abnormal and flat(E’). Many ommatidia are observed at PR apical level, but they frequently lack full complement of photoreceptors, and have abnormal looking rhabdomeres(E’’). These abnormal ommatidia do not extend depth of the eye, as seen by pigment rim like appearance in PR basal sections (E’’’).

F: Extreme degeneration phenotype of *Pros-Gal4; UAS-Arm** eyes.

G-H: At the periphery of *Pros-Gal4; UAS-Arm** eyes, we observe a much thicker pigment rim (marked by black bar) as compared to a wild type pigment rim (H).

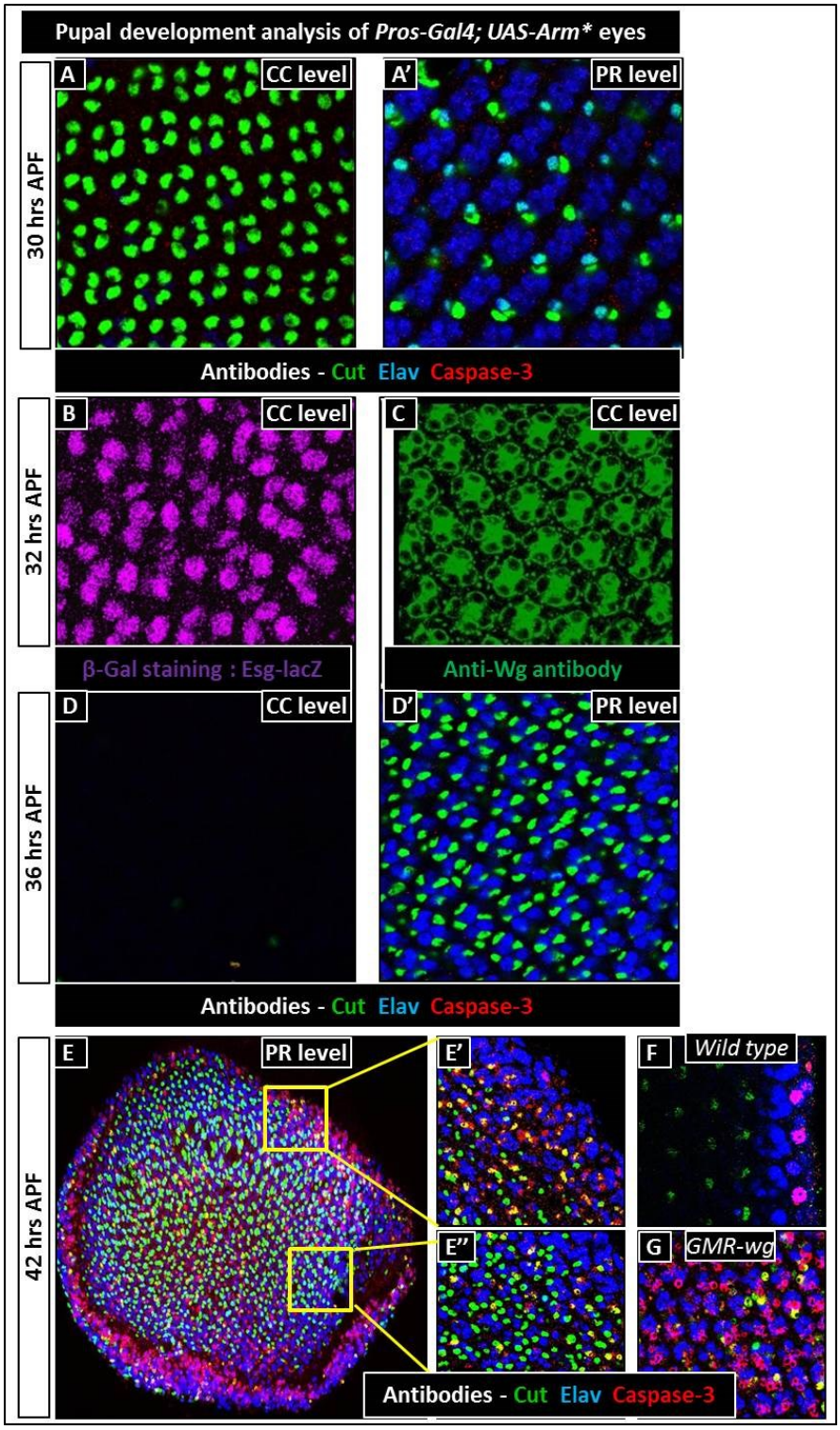
Pupal development analysis of *Pros-Gal4; UAS-Arm** eyes
30 hrs APF
A
CC level
A'
PR level
Antibodies - Cut Elav Caspase-3
32 hrs APF
B
CC level
C
CC level
β-Gal staining : Esg-lacZ
Anti-Wg antibody
36 hrs APF
D
CC level
D'
PR level
Antibodies - Cut Elav Caspase-3
42 hrs APF
E
PR level
E'
E''
F
Wild type
G
GMR-wg
Antibodies - Cut Elav Caspase-3

Figure 13: Effects of activating Wg transduction in the cone cells on pupal development of the eye

A,A': *Pros-Gal4; UAS-Arm** eyes show wild type pupal development until 30 hrs APF.

B, C: At 32hrsAPF, all the cone cells express Esg (as evidenced by expression of the transcriptional reporter *Esg-LacZ*-B)and by anti-Wg antibody(C).

D, D': At 36hrsAPF, all the cone cells collapse to the PR level, similar to *GMR-wg* eye development.

E: AT 42hrsAPF, apoptosis occurs in a broad peripheral swathe of ommatidia unlike wild type eyes(F). Outermost rows of ommatidia in *Pros-Gal4; UAS-Arm** eyes undergo apoptosis(E') similar to *GMR-wg* eyes(G) while the main body ommatidia show a low level sporadic apoptosis(E'').

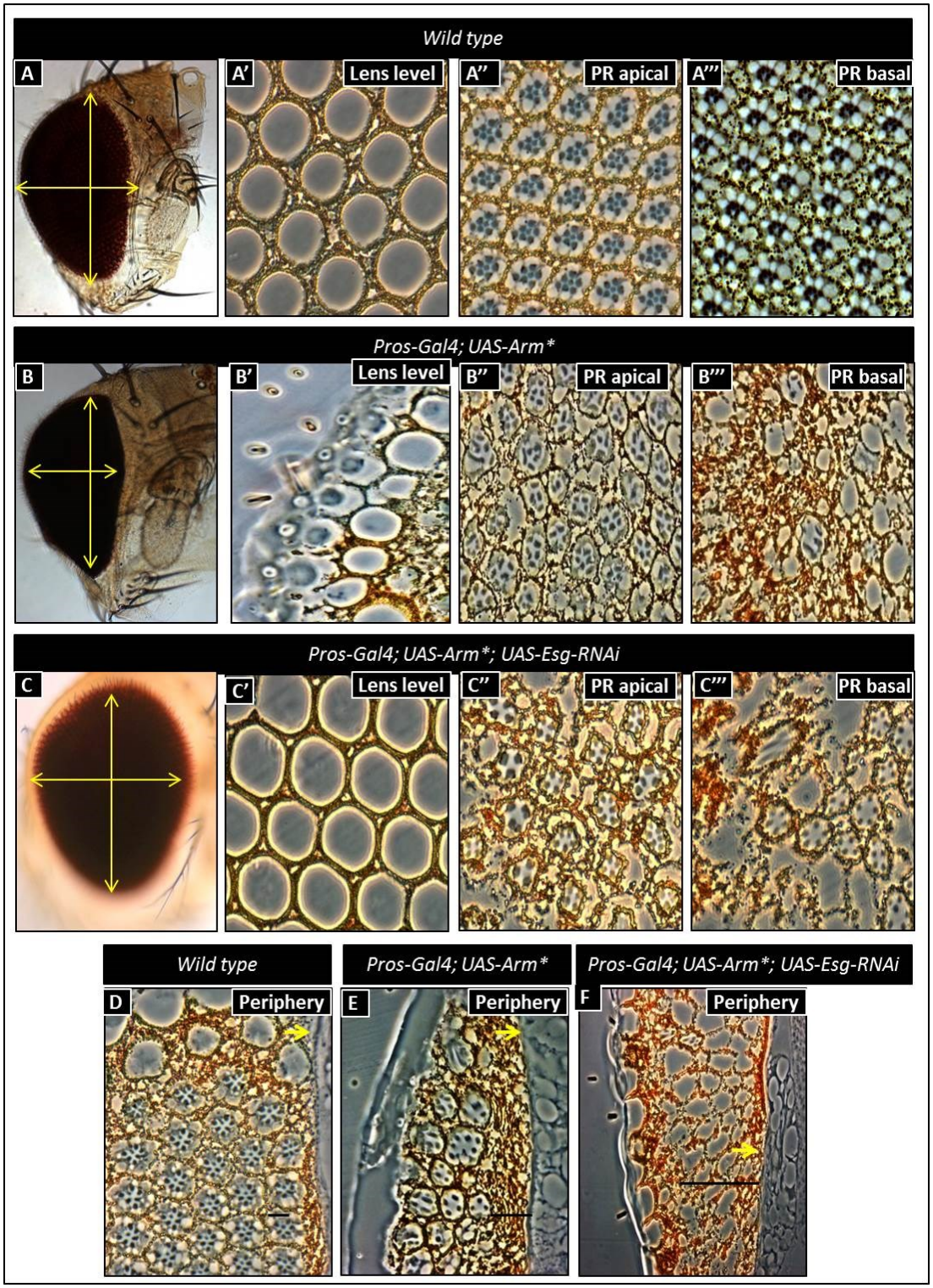
Wild type
A
A'
Lens level
A''
PR apical
A'''
PR basal
Pros-Gal4; UAS-Arm*
B
B'
Lens level
B''
PR apical
B'''
PR basal
Pros-Gal4; UAS-Arm*; UAS-Esg-RNAi
C
C'
Lens level
C''
PR apical
C'''
PR basal
Wild type
Pros-Gal4; UAS-Arm*
Pros-Gal4; UAS-Arm*; UAS-Esg-RNAi
D
Periphery
E
Periphery
F
Periphery

Figure 14: Removing Escargot from the cone cells prevents their collapse and apoptosis.

A-A’’’: Wild type adult eye and sections showing normal array of lenses and photoreceptors.

B-B’’’: *Pros-Gal4; UAS-Arm** eyes. The lenses are abnormal, and the underlying ommatidia are frequently incomplete and stunted.

C-C’’’: *Pros-Gal4; UAS-Arm*; UAS-Esg-RNAi* eyes. The eye size is restored to wild type(C), and the lens array is restored to wild type appearance (C’). The ommatidia underneath are still incomplete and stunted(C’’-C’’’).

D-F: Peripheral sections show that instead of the thicker pigment rim (marked by black bar) of *Pros-Gal4; UAS-Arm** eyes, the *Pros-Gal4; UAS-Arm*; UAS-Esg-RNAi* eyes show an empty meshwork of ommatidia adjacent to the HC (marked by yellow arrows).

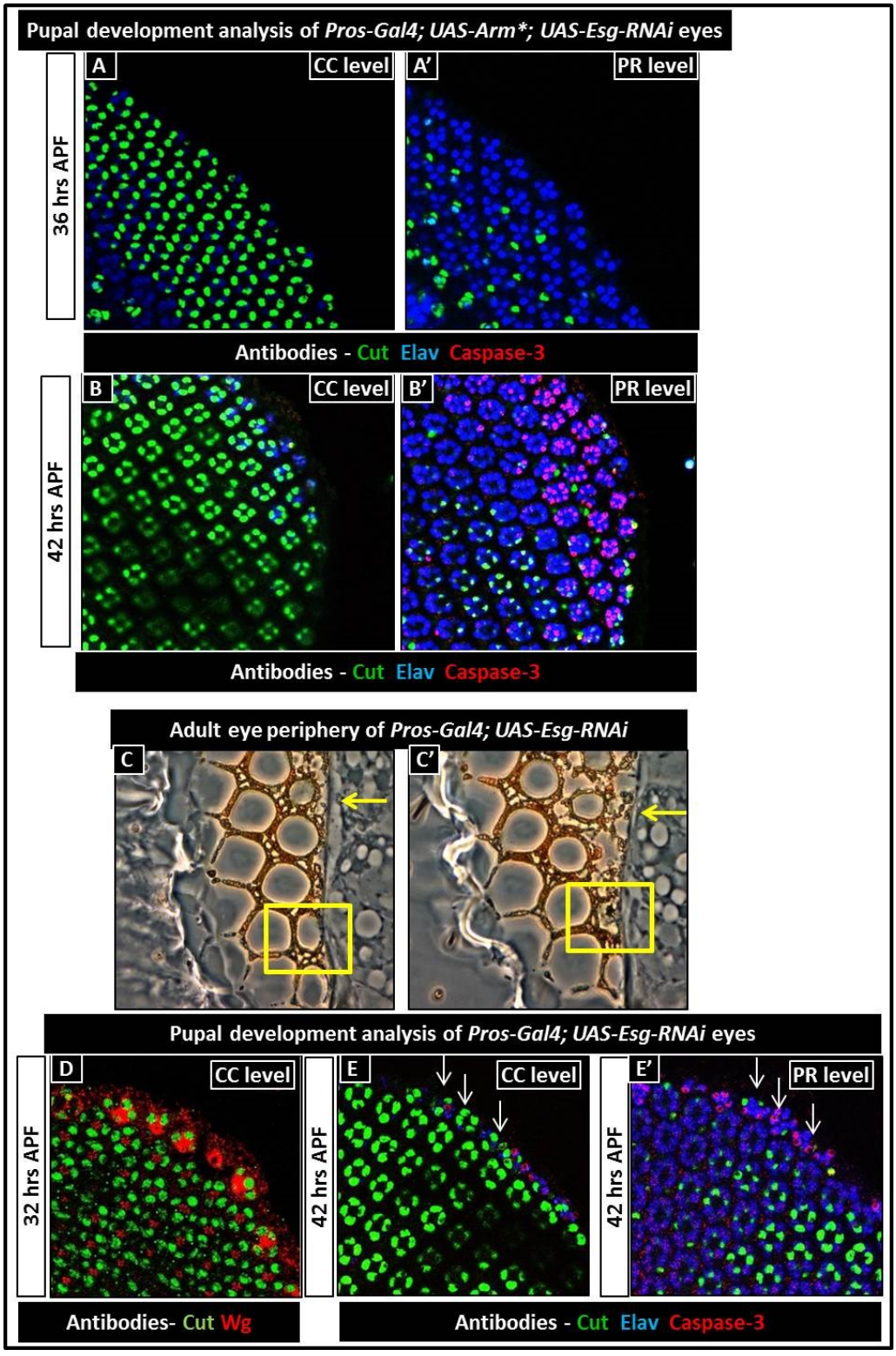
Pupal development analysis of *Pros-Gal4; UAS-Arm*; UAS-Esg-RNAi* eyes
A
CC level
A'
PR level
36 hrs APF
Antibodies - Cut Elav Caspase-3
B
CC level
B'
PR level
42 hrs APF
Antibodies - Cut Elav Caspase-3
Adult eye periphery of *Pros-Gal4; UAS-Esg-RNAi*
C
C'
Pupal development analysis of *Pros-Gal4; UAS-Esg-RNAi* eyes
D
CC level
32 hrs APF
Antibodies- Cut Wg
E
CC level
42 hrs APF
E'
PR level
42 hrs APF
Antibodies - Cut Elav Caspase-3

Figure 15: Effect of removal of Escargot on pupal eye development

A-A': The cone cells of *Pros-Gal4; UAS-Arm*; UAS-Esg-RNAi* eyes do not collapse to PR level at 36hrsAPF.

B-B': The cone cells stay at their apical positions(B), while underlying photoreceptors undergo apoptosis in a broad outer swathe (B').

C-C': Adult eye periphery of *Pros-Gal4; UAS-Esg-RNAi* eyes show the presence of small lenses (yellow box) adjacent to HC(marked by yellow arrow), deeper sections show absence of underlying photoreceptors(C').

D-E': Pupal development of *Pros-Gal4; UAS-Esg-RNAi* eye periphery

D: Peripheral cone cells express Wg at 32hrsAPF.

E-E': At 42hrsAPF, the peripheral cone cells are present apically while the underlying photoreceptors undergo apoptosis (white arrows).

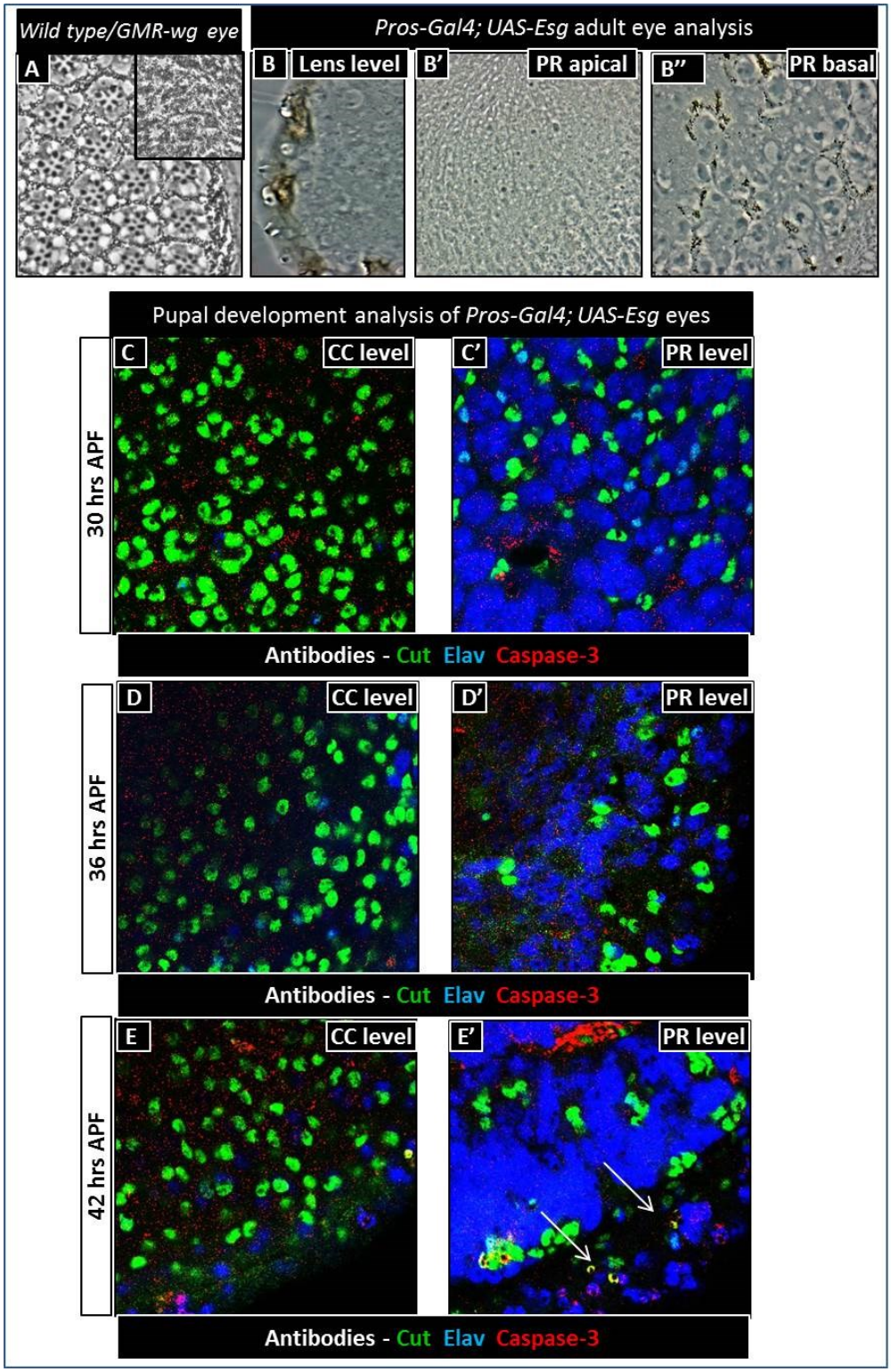
Wild type/GMR-wg eye
A
Pros-Gal4; UAS-Esg adult eye analysis
B
Lens level
B'
PR apical
B''
PR basal
Pupal development analysis of Pros-Gal4; UAS-Esg eyes
30 hrs APF
C
CC level
C'
PR level
Antibodies - Cut Elav Caspase-3
36 hrs APF
D
CC level
D'
PR level
Antibodies - Cut Elav Caspase-3
42 hrs APF
E
CC level
E'
PR level
Antibodies - Cut Elav Caspase-3

Figure 16: Effect of ectopic expression of Escargot in the cone cells of the eye.

A: Section through adult wild type eye. Inset shows section through *GMR-wg* eye.

B-B’’’: Sections through *Pros-Gal4; UAS-Esg* adult eyes. Lenses are necrotic(B), the apical sections at PR level are *GMR-wg* like in appearance. Deeper sections through the retina show photoreceptor remnants collapsed onto basal lamina(B’’’).

C-E’: Pupal development of *Pros-Gal4; UAS-Esg* eyes.

C-C’: Cone cells show a precocious collapse to PR level as early as 30hrsAPF.

D-D’: Cone cells continue to randomly collapse as the photoreceptors appear to clump together.

E-E’: At 42hrs APF, the cone cells and photoreceptors are clumped together, there is still some evidence of peripheral apoptosis(white arrows).

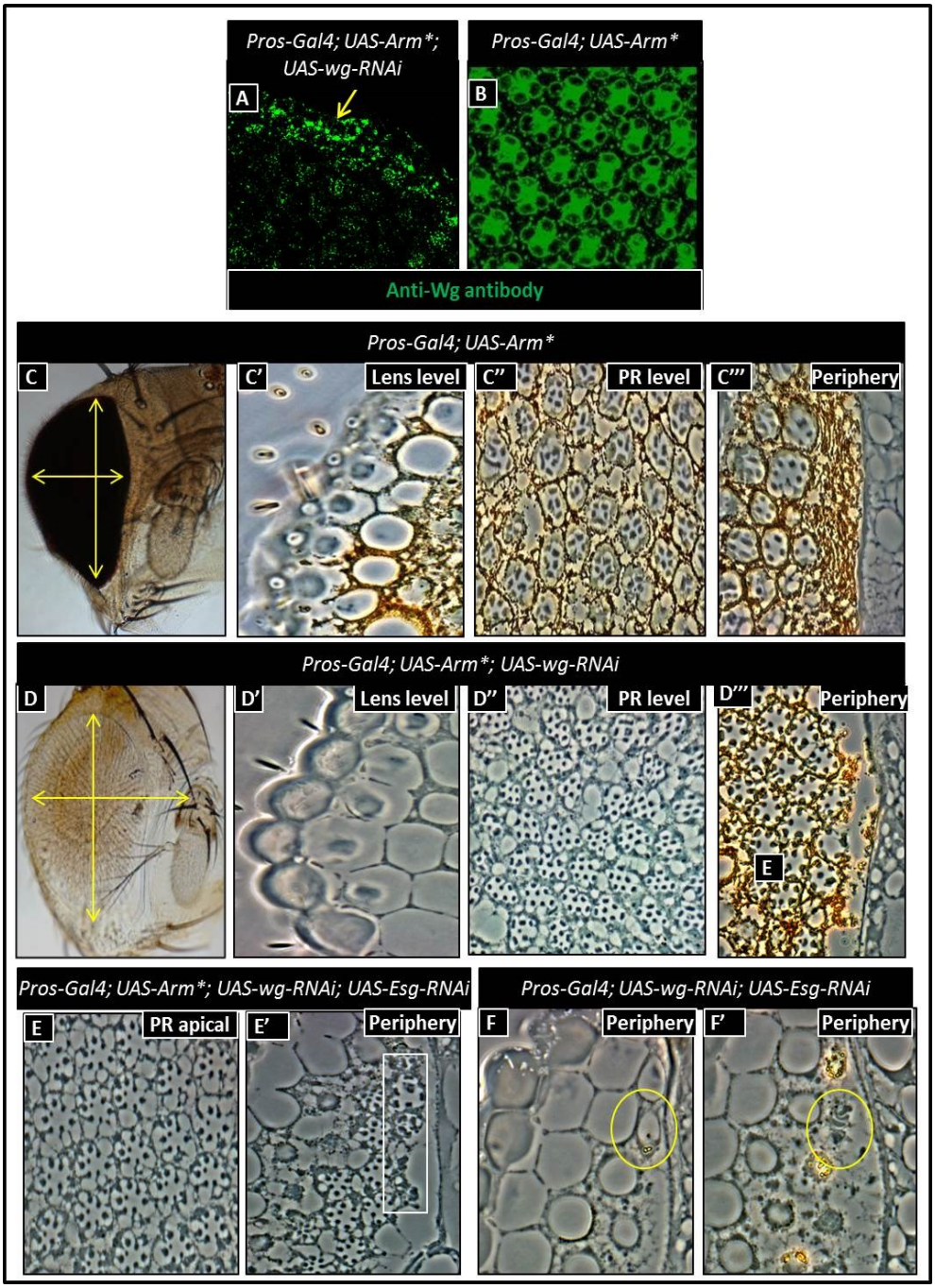
Pros-Gal4; UAS-Arm*; UAS-wg-RNAi
A
Pros-Gal4; UAS-Arm*
B
Anti-Wg antibody
Pros-Gal4; UAS-Arm*
C
C'
Lens level
C''
PR level
C'''
Periphery
Pros-Gal4; UAS-Arm*; UAS-wg-RNAi
D
D'
Lens level
D''
PR level
D'''
Periphery
E
Pros-Gal4; UAS-Arm*; UAS-wg-RNAi; UAS-Esg-RNAi
E
PR apical
E'
Periphery
Pros-Gal4; UAS-wg-RNAi; UAS-Esg-RNAi
F
Periphery
F'
Periphery

Figure 17: Effect of removal of secondary Wg from the cone cells on peripheral apoptosis

A-B: At 32hrsAPF, the cone cells of *Pros-Gal4; UAS-Arm*; UAS-Wg RNAi* eyes show extremely reduced Wg expression as compared to *Pros-Gal4; UAS-Arm** eyes(B).

C-C’’’: Adult eyes and sections of *Pros-Gal4; UAS-Arm**

D-D’’’: Adult eyes and sections of *Pros-Gal4; UAS-Arm*; UAS-Wg-RNAi*

These eyes are slightly larger in size (D). The lenses are considerably improved in appearance(D’). The underlying ommatidia are relatively more normal in appearance (D’’). The peripheral section shows presence of ommatidia, close to a normal pigment rim (D’’’) as opposed to thick pigment rim of *Pros-Gal4; UAS-Arm** (C’’’).

E-E’: Removal of both Wg and Esg from *Pros-Gal4; UAS-Arm** eyes

The sections through the eyes show an almost wild type ommatidial array (E), peripheral section shows presence of incomplete ommatidia adjacent to the HC (E’)(marked by white box)

F-F’: removal of Esg and Wg from the cone cells in a wild type periphery shows the presence of incomplete ommatidia adjacent to the HC (marked by yellow circles).

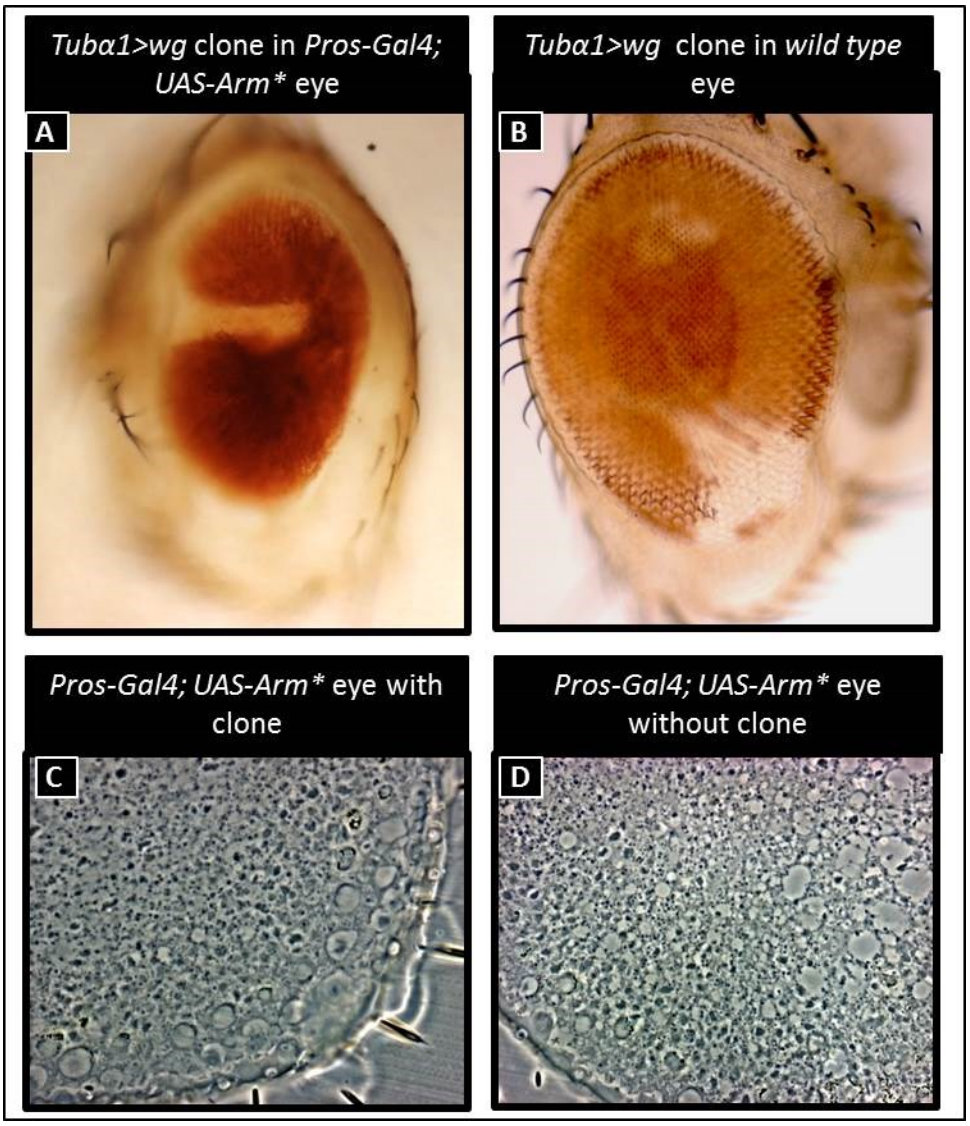

Figure 18: Clonal addition of tonic levels of Wg to *Pros-Gal4; UAS-Arm** eyes

A: Tubα1>*wg* clone in *Pros-Gal4; UAS-Arm** eye produces smooth scar externally.

B: Tubα1>*wg* clone in a wild type eye causes balding, as seen in the white region of the eye.

C: Section through the *Pros-Gal4; UAS-Arm** eye does not show a significant difference in appearance from the control eye lacking the clone (D).

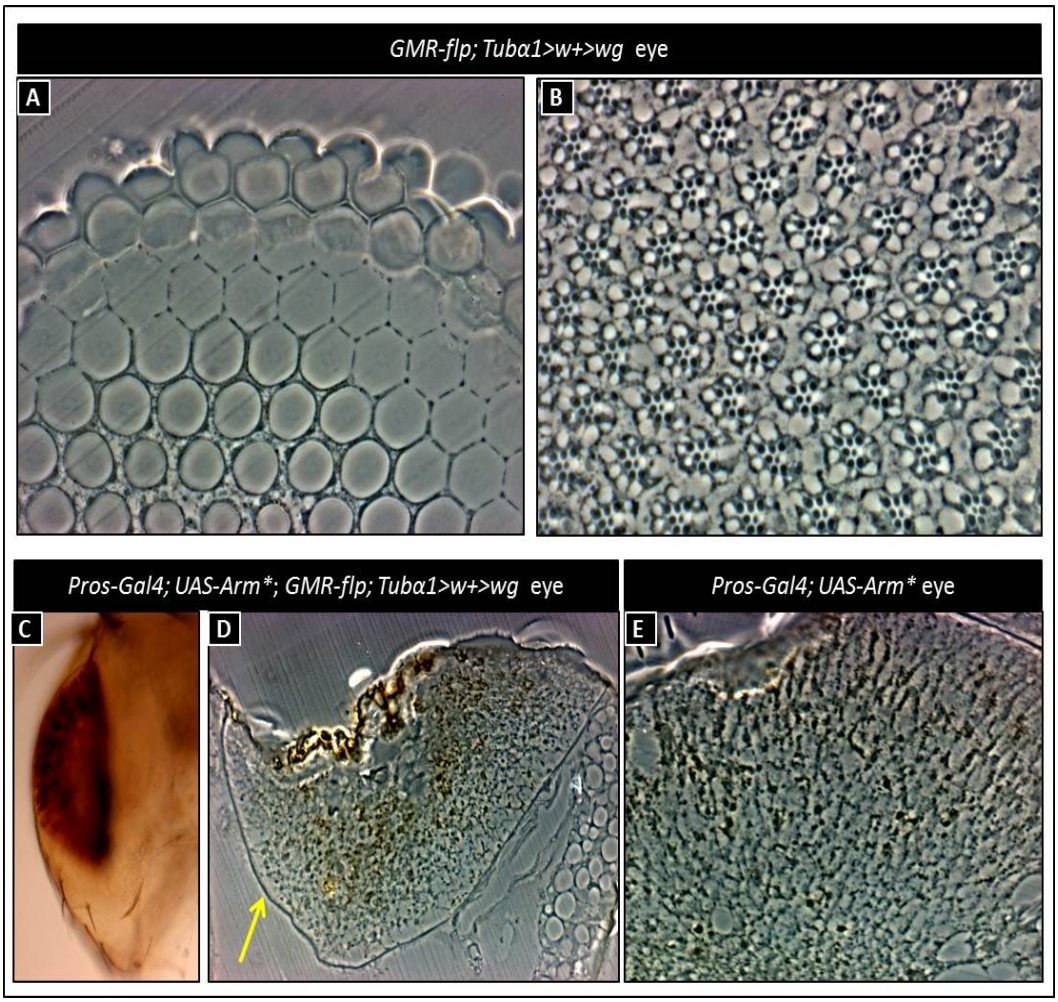

Figure 19: Addition of tonic levels of Wg to *Pros-Gal4; UAS-Arm** eyes using *GMR-flp.*

A: Adult eye of *GMR-flp; Tubα1>w+>wg* genotype. The lenses are wild-type but there are no bristles.

B: Section through *GMR-flp; Tubα1>w+>wg* eyes shows normal ommatidial array.

C: Adult eye of *Pros-Gal4; UAS-Arm*; GMR-flp; Tubα1>w+>wg* – small *GMR-wg* like eye.

D: Section through *Pros-Gal4; UAS-Arm*; GMR-flp; Tubα1>w+>wg* eyes show very small retinal field, no ommatidial structure evident.

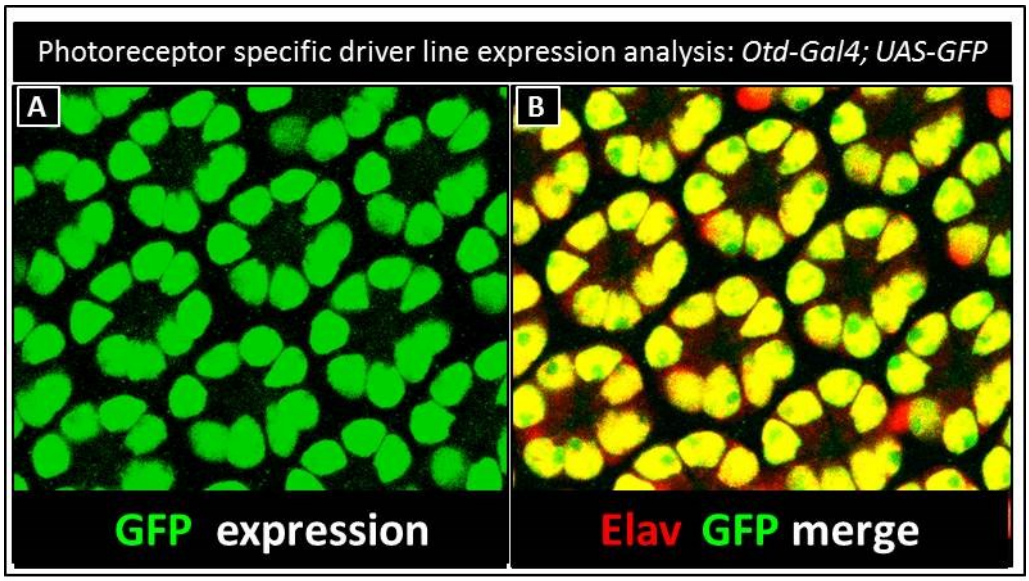

Figure 20: Expression of photoreceptor cell specific driver line: *Otd-Gal4*.

A: *UAS-GFP* driven by *Otd-Gal4* shows expression only in the photoreceptor cells at this focal plane, as evidenced by GFP antibody.

B: Merged image panel showing Elav (photoreceptor marker) with *UAS-GFP* expression.

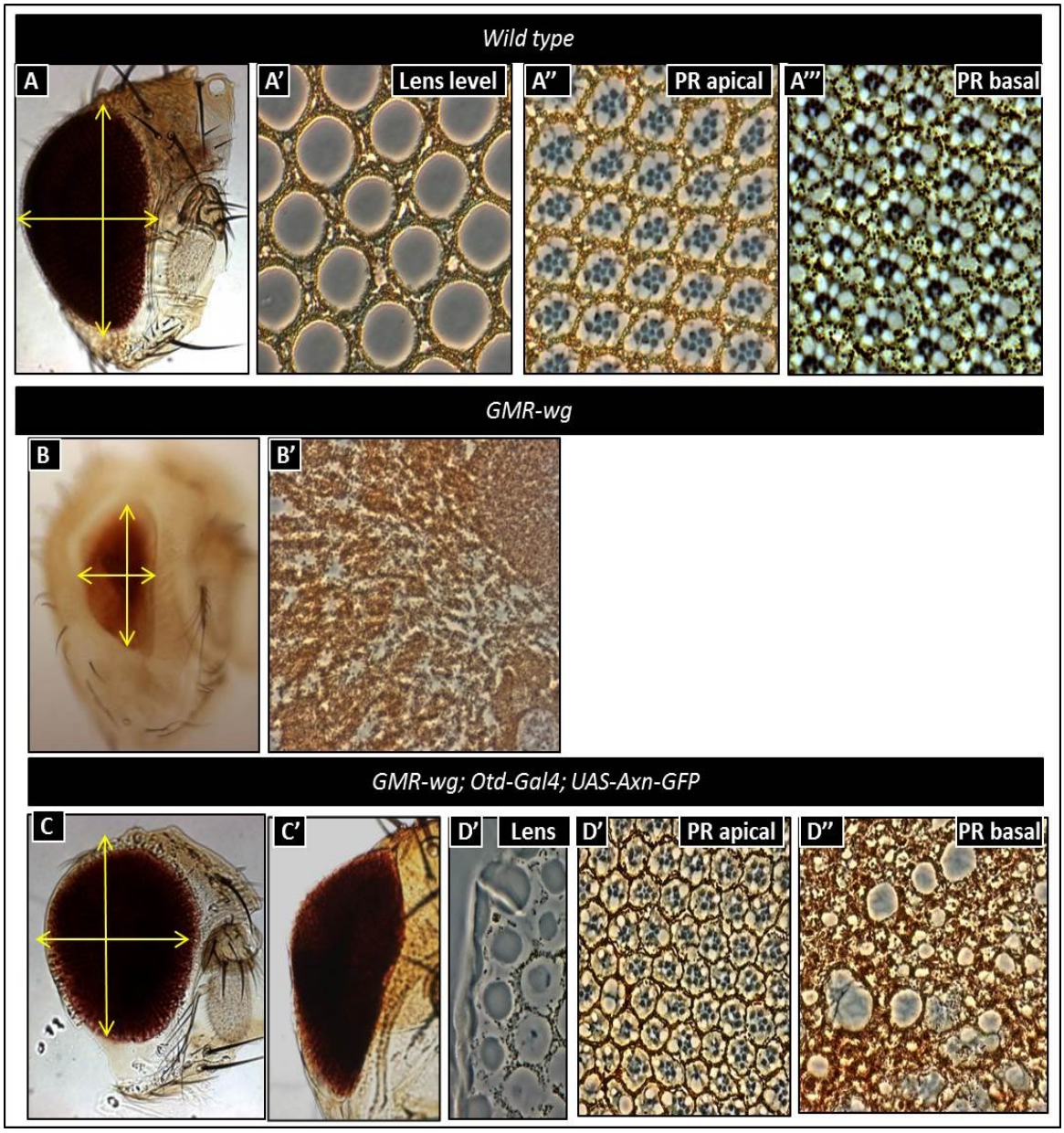

Figure 21: Blocking Wg transduction in the photoreceptors leads to a huge rescue of *GMR-wg* induced pseudo periphery eye

A-A''': Wild type eye, and the sections show normal array of ommatidia at the lens level and at the PR level.

B-B': *GMR-wg* eye, sections show pseudo periphery tissue full of pigment cells.

C-D'': *GMR-wg; Otd-Gal4; UAS-Axn-GFP* eyes. There is a partial rescue of size, but the eye still lacks bristle s(orange box, C'). Sections through lens level (D) shows abnormal lenses, but at the PR level (D') we see significant restoration of ommatidia. These ommatidia frequently have an incomplete set of photoreceptors, and fail to extend the depth of the retina(D'').

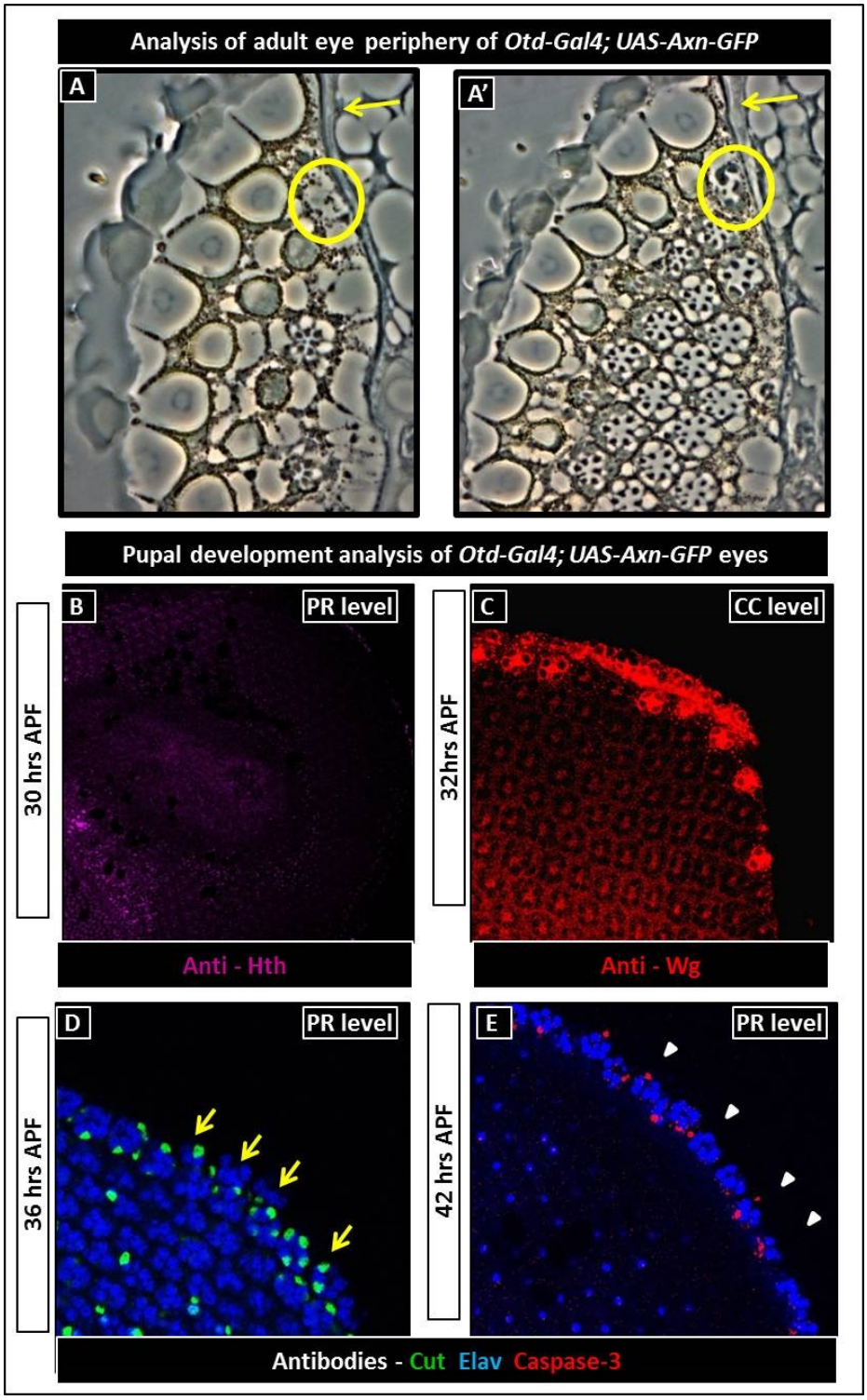
Analysis of adult eye periphery of Otd-Gal4; UAS-Axn-GFP
A
A'
Pupal development analysis of Otd-Gal4; UAS-Axn-GFP eyes
30 hrs APF
B
PR level
Anti - Hth
32hrs APF
C
CC level
Anti - Wg
36 hrs APF
D
PR level
42 hrs APF
E
PR level
Antibodies - Cut Elav Caspase-3

Figure 22: Effect of removing Wg transduction from the photoreceptors at the periphery.

A, A': Adult eye periphery of *Otd-Gal4; UAS-Axn-GFP* show the presence of small ommatidia (yellow circle) adjacent to the HC (marked by yellow arrow) (A'), but no lenses in the apical sections(A).

B: At 27hrsAPF, there is no Hth expression (in pink) in the pupae.

C: Wg is expressed at the periphery at 32hrsAPF(shown in red).

D: Yellow arrows indicate the peripheral cone cells collapse to the level of photoreceptors

E: At 42hrsAPF, apoptosis is reduced at the periphery (white arrowheads).

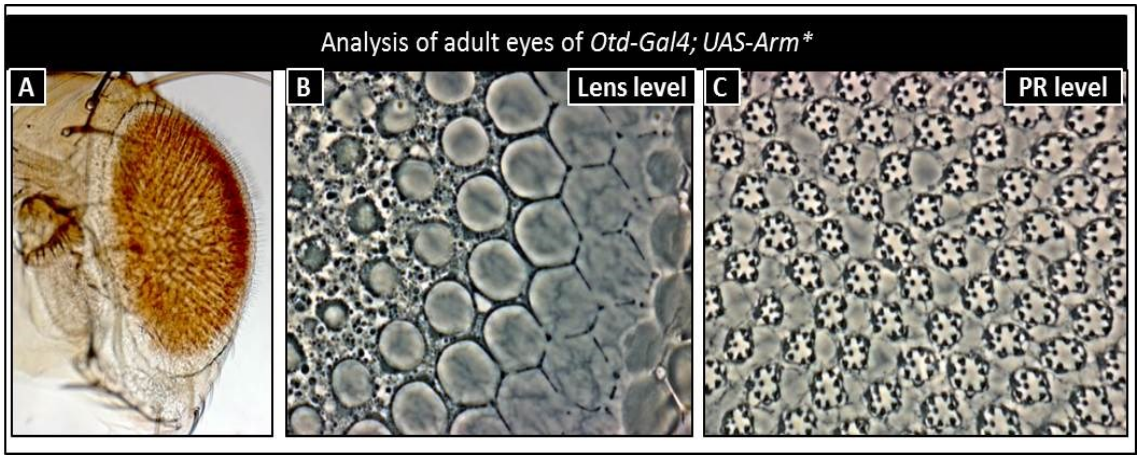

Figure 23: Ectopic activation of Wg signaling in photoreceptors has no effect.

This panel shows *Otd-Gal4; UAS-Arm** eyes

A: Adult eye looks wild type

B: Apical sections show normal lenses and deeper sections (C) show wild type ommatidial array.

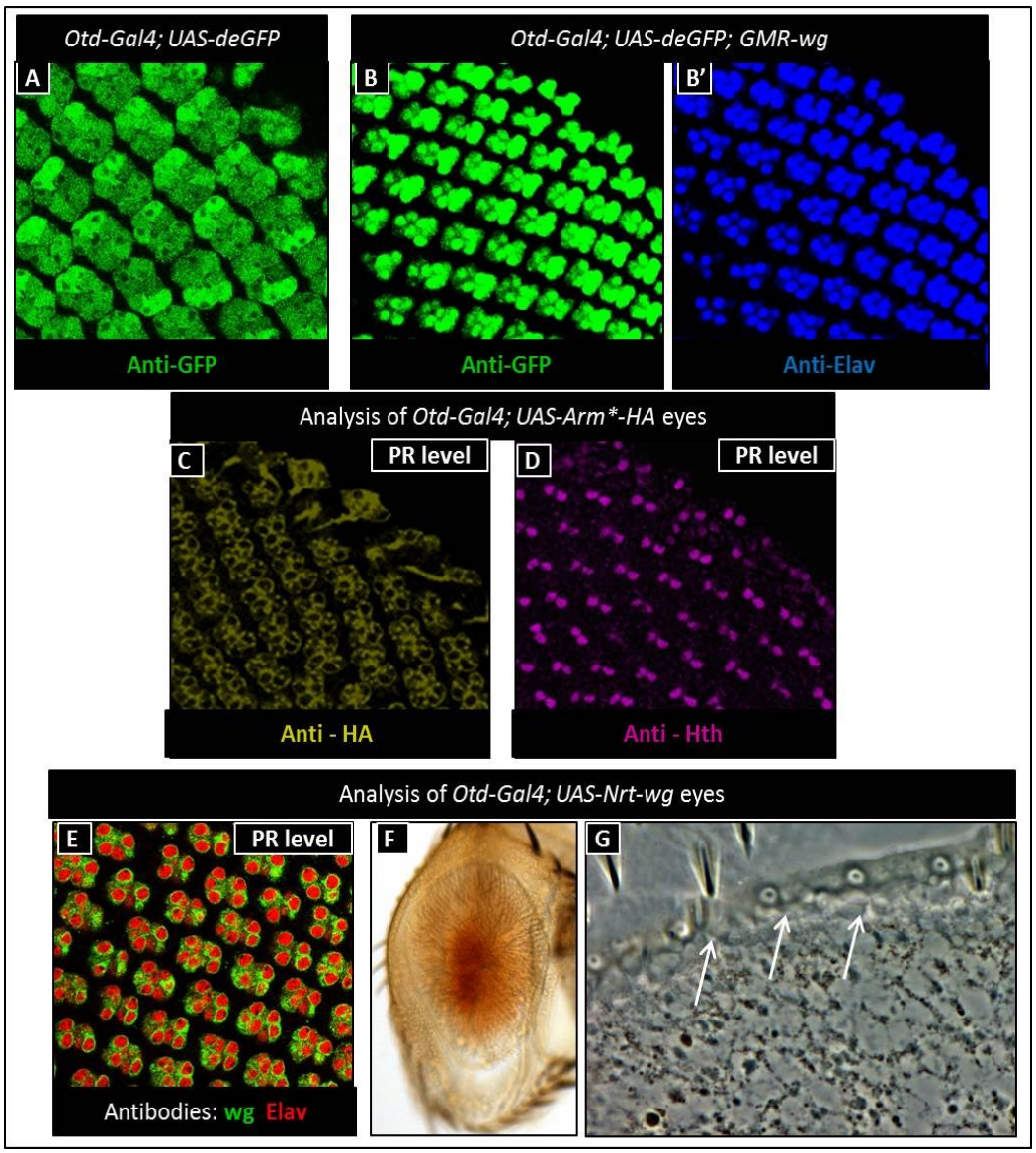
Otd-Gal4; UAS-deGFP
A
Anti-GFP
Otd-Gal4; UAS-deGFP; GMR-wg
B
Anti-GFP
B'
Anti-Elav
Analysis of Otd-Gal4; UAS-Arm*-HA eyes
C
PR level
Anti - HA
D
PR level
Anti - Hth
Analysis of Otd-Gal4; UAS-Nrt-wg eyes
E
PR level
Antibodies: wg Elav
F
G

Figure 24: Can Wg signaling be activated in the photoreceptors using *Otd-Gal4*?

A: *Otd-Gal4; UAS-deGFP* pupal retinas show GFP expression throughout pupal development.

B: *GMR-Wg; Otd-Gal4; UAS-deGFP* pupal retinas show strong expression throughout pupal development. This expression is restricted to photoreceptors, as compared with Elav expression in blue (B').

C: *Otd-Gal4; UAS-Arm*-HA* pupal eyes show expression of anti-HA antibody (in yellow) in all the photoreceptors

D: *Otd-Gal4; UAS-Arm*-HA* pupal eyes show expression of Hth (in pink) in the central photoreceptors in the dorsal half of the eye.

E: *Otd-gal4; UAS-Nrt-wg* pupal eyes show Wg expression(in green) at the membranes of the photoreceptors(marked by Elav in red).

F: *Otd-gal4; UAS-Nrt-Wg* adult eyes are small, with no lenses and a rough array of bristles.

G: Sections through *Otd-Gal4; UAS-Nrt-Wg* eyes show necrotic lenses (white arrows) and a complete absence of all ommatidial structures.

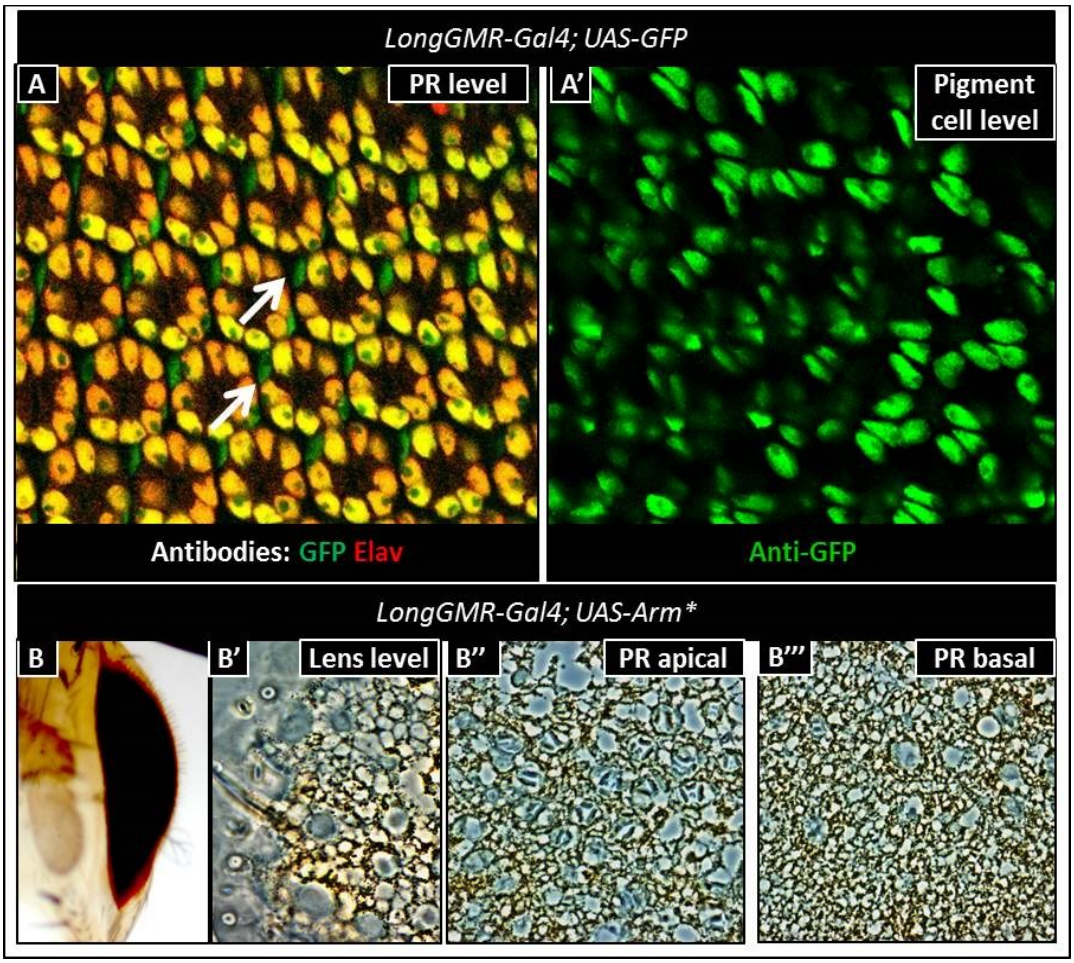

Figure 25: Ectopic expression analysis using *LongGMR-Gal4* driver line

A,A': Analysis of the expression pattern of *LongGMRGal4* using *UAS-GFP* shows expression in the photoreceptors (co-stained with Elav in red), and in the primary pigment cells(white arrows, A) and secondary/tertiary pigment cells(A').

B-B''': Adult eyes of *LongGMR-Gal4; UAS-Arm**

These eyes are smaller in size than a wild type eye, and have distorted abnormal lenses(B'). The photoreceptors appear distorted and stunted(B''-B'''). The pigment cell lattice is also disarrayed.

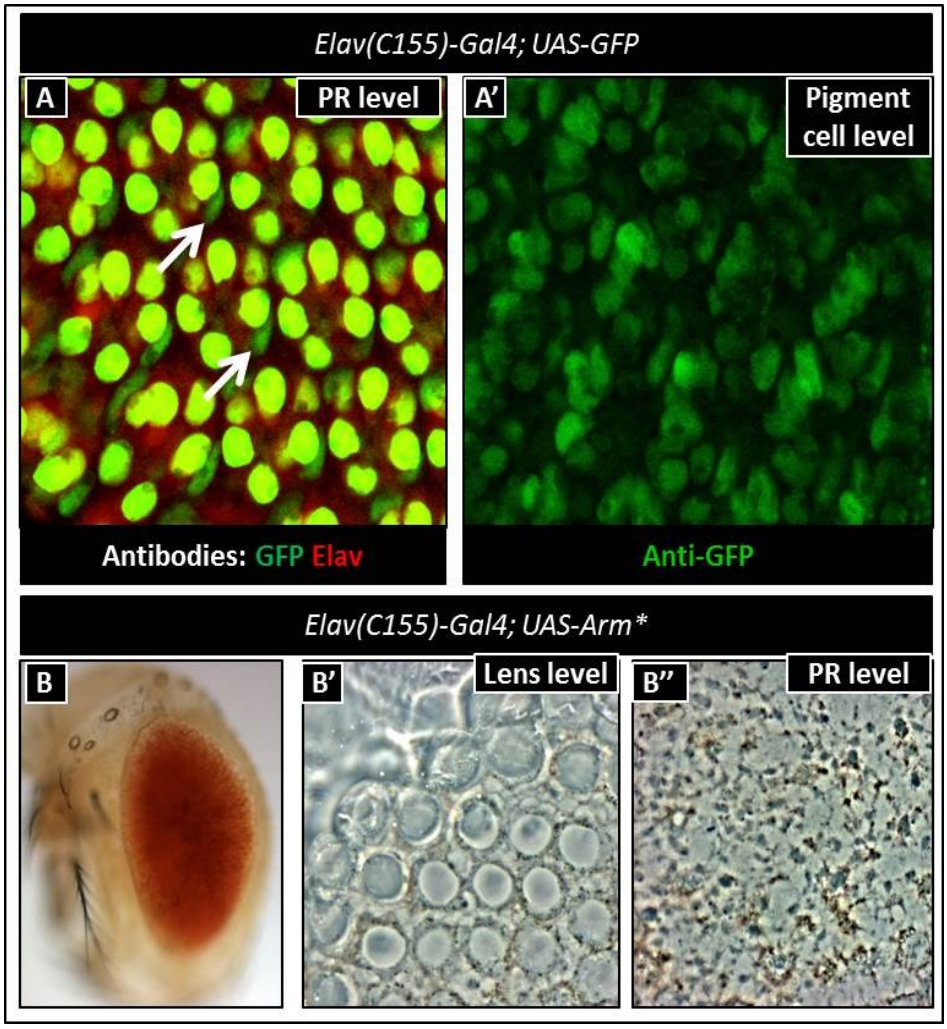

Figure 26: Ectopic expression analysis using *Elav(C155)-Gal4* driver line

A,A': Analysis of the expression pattern of *Elav(C155)-Gal4* using *UAS-GFP* shows expression in the photoreceptors (co-stained with Elav in red), and in the primary pigment cells(white arrows, A) and secondary/tertiary pigment cells(A').

B-B'': Adult eyes of *Elav(C155)Gal4; UAS-Arm**

These eyes are smaller in size(B) than a wild type eye, and have fewer, albeit wild type looking lenses (B'). Deeper sections through the retina show a complete degeneration of photoreceptors and a disarrayed pigment cell lattice (B'').

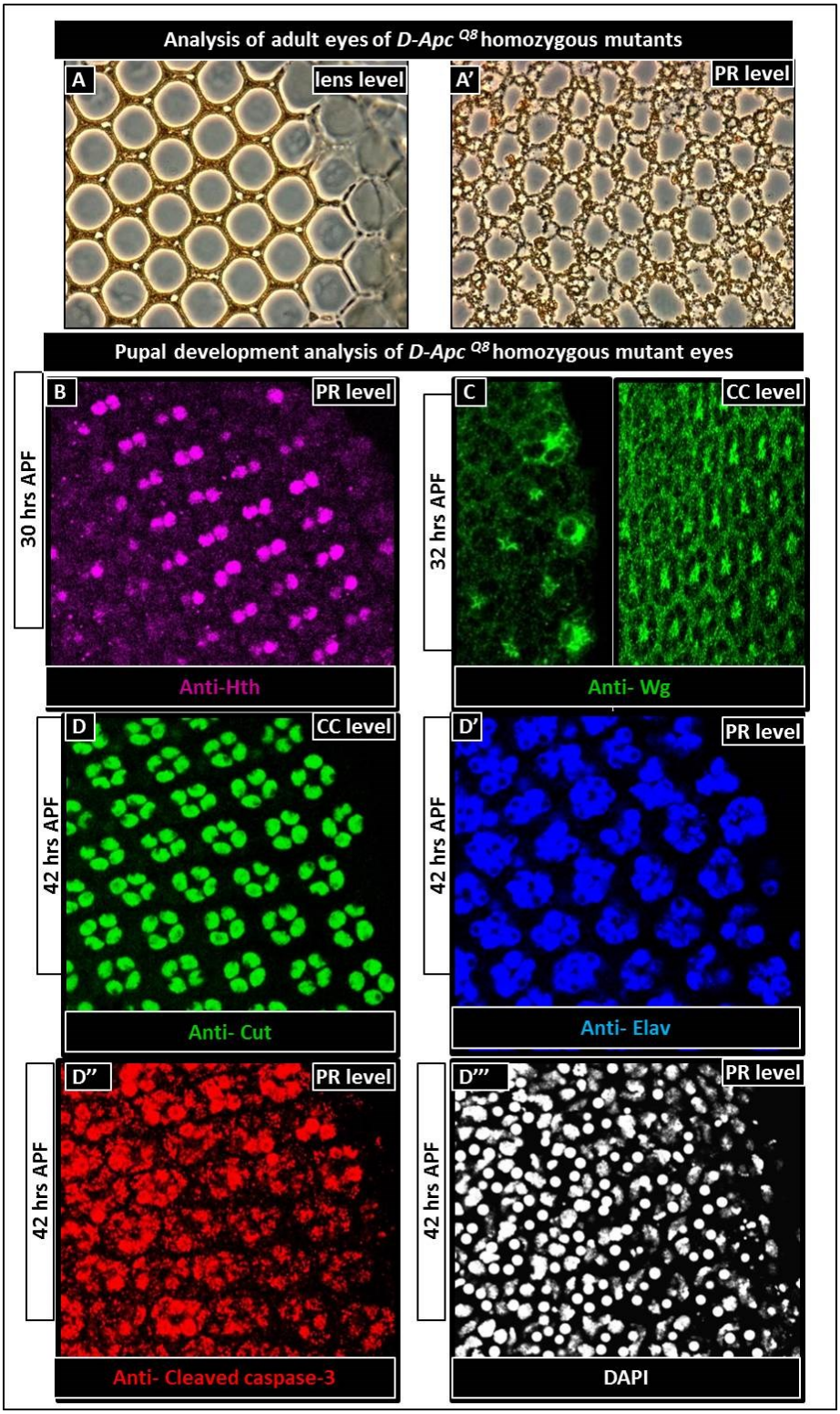
Analysis of adult eyes of D-Apc Q8 homozygous mutants
A
lens level
A'
PR level
Pupal development analysis of D-Apc Q8 homozygous mutant eyes
30 hrs APF
B
PR level
Anti-Hth
32 hrs APF
C
CC level
Anti- Wg
42 hrs APF
D
CC level
Anti- Cut
42 hrs APF
D'
PR level
Anti- Elav
42 hrs APF
D''
PR level
Anti- Cleaved caspase-3
42 hrs APF
D'''
PR level
DAPI

Figure 27: Analysis of *D-Apc*^{Q8} homozygous mutant eyes

A, A': Section through adult eyes show perfect lenses in the apical section (A) and complete absence of photoreceptors in the more basal section (A') although the pigment cell lattice is intact.

B: Expression of Hth(marked in pink) at 28hrsAPF in all central photoreceptors of dorsal region

C: At 32hrs APF, strong expression of Wg(marked in green) in peripheral cone cells as compared to main body cone cells(B')

D-D''': These 4 panels show the pupal retina at 42hrsAPF. Cone cells (marked by Cut in green) are intact(D), while photoreceptors (marked by Elav in blue) are undergoing apoptosis(D'). This apoptosis is visualized by caspase staining(in red)(D'') and by DAPI staining in grey (D''')

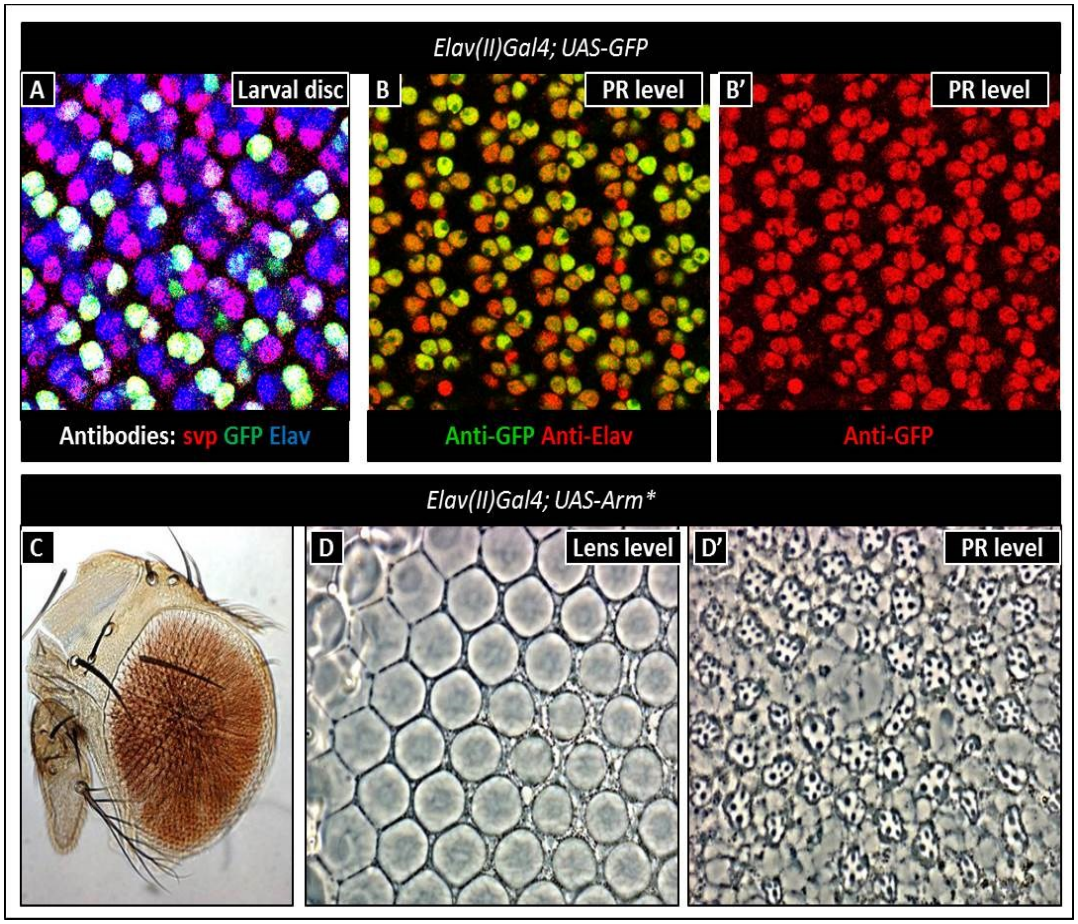

Figure 28: *Elav(II)Gal4; UAS-Arm**can cause photoreceptor degeneration.

A: Expression analysis of *Elav(II)Gal4* using *UAS-GFP* shows strong expression in the larval stages in R3/R4.

B, B': expression at pupal stages is restricted to photoreceptors, as indicated by co-staining with Elav(in red).

C-D': Adult eyes of *Elav(II)Gal4; UAS-Arm**

C: Externally these eyes look wild type.

D: Apical sections show normal lenses

D': Pigment cell lattice is disturbed with ommatidia frequently missing photoreceptors.

Ahmed, Y., S. Hayashi, A. Levine and E. Wieschaus (1998). "Regulation of armadillo by a Drosophila APC inhibits neuronal apoptosis during retinal development." Cell 93(7): 1171-1182.

Ashraf, S. I., X. Hu, J. Roote and Y. T. Ip (1999). "The mesoderm determinant snail collaborates with related zinc-finger proteins to control Drosophila neurogenesis." EMBO J **18**(22): 6426-6438.

Bergmann, A., J. Agapite, K. McCall and H. Steller (1998). "The Drosophila gene hid is a direct molecular target of Ras-dependent survival signaling." Cell **95**(3): 331-341.

Boulay, J. L., C. Dennefeld and A. Alberga (1987). "The Drosophila developmental gene snail encodes a protein with nucleic acid binding fingers." Nature **330**(6146): 395-398.

Brand, A. H. and N. Perrimon (1993). "Targeted gene expression as a means of altering cell fates and generating dominant phenotypes." Development **118**(2): 401-415.

Cadigan, K. M., A. D. Jou and R. Nusse (2002). "Wingless blocks bristle formation and morphogenetic furrow progression in the eye through repression of Daughterless." Development **129**(14): 3393-3402.

Cagan, R. L. and D. F. Ready (1989). "The emergence of order in the Drosophila pupal retina." Dev Biol

136(2): 346-362.

Clem, R. J., M. Fechheimer and L. K. Miller (1991). "Prevention of apoptosis by a baculovirus gene during infection of insect cells." Science **254**(5036): 1388-1390.

Cliffe, A., F. Hamada and M. Bienz (2003). "A role of Dishevelled in relocating Axin to the plasma membrane during wingless signaling." Curr Biol **13**(11): 960-966.

Fan, Y. and A. Bergmann (2010). "The cleaved-Caspase-3 antibody is a marker of Caspase-9-like DRONC activity in Drosophila." Cell Death Differ **17**(3): 534-539.

Golic, K. G. and S. Lindquist (1989). "The FLP recombinase of yeast catalyzes site-specific recombination in the Drosophila genome." Cell **59**(3): 499-509.

Goyal, L., K. McCall, J. Agapite, E. Hartwieg and H. Steller (2000). "Induction of apoptosis by Drosophila reaper, hid and grim through inhibition of IAP function." EMBO J **19**(4): 589-597.

Grether, M. E., J. M. Abrams, J. Agapite, K. White and H. Steller (1995). "The head involution defective gene of Drosophila melanogaster functions in programmed cell death." Genes Dev **9**(14): 1694-1708.

Hart, M. J., R. de los Santos, I. N. Albert, B. Rubinfeld and P. Polakis (1998). "Downregulation of beta-catenin by human Axin and its association with the APC tumor suppressor, beta-catenin and GSK3 beta." Curr Biol **8**(10): 573-581.

Hay, B. A., D. A. Wassarman and G. M. Rubin (1995). "Drosophila homologs of baculovirus inhibitor of apoptosis proteins function to block cell death." Cell **83**(7): 1253-1262.

Hay, B. A., T. Wolff and G. M. Rubin (1994). "Expression of baculovirus P35 prevents cell death in Drosophila." Development **120**(8): 2121-2129.

Hayashi, T., C. Xu and R. W. Carthew (2008). "Cell-type-specific transcription of prospero is controlled by combinatorial signaling in the Drosophila eye." Development **135**(16): 2787-2796.

Ikeda, S., S. Kishida, H. Yamamoto, H. Murai, S. Koyama and A. Kikuchi (1998). "Axin, a negative regulator of the Wnt signaling pathway, forms a complex with GSK-3beta and beta-catenin and promotes GSK-3beta-dependent phosphorylation of beta-catenin." EMBO J **17**(5): 1371-1384.

Kimelman, D. and W. Xu (2006). "beta-catenin destruction complex: insights and questions from a structural perspective." Oncogene **25**(57): 7482-7491.

Lieber, T., S. Kidd and G. Struhl (2011). "DSL-Notch signaling in the Drosophila brain in response to olfactory stimulation." Neuron **69**(3): 468-481.

Lim, H. Y. and A. Tomlinson (2006). "Organization of the peripheral fly eye: the roles of Snail family transcription factors in peripheral retinal apoptosis." Development **133**(18): 3529-3537.

Lin, D. M. and C. S. Goodman (1994). "Ectopic and increased expression of Fasciclin II alters motoneuron growth cone guidance." Neuron **13**(3): 507-523.

Lin, H. V., A. Rogulja and K. M. Cadigan (2004). "Wingless eliminates ommatidia from the edge of the developing eye through activation of apoptosis." Development **131**(10): 2409-2418.

Ma, C. and K. Moses (1995). "Wingless and patched are negative regulators of the morphogenetic furrow and can affect tissue polarity in the developing Drosophila compound eye." Development **121**(8): 2279-2289.

Mendoza-Topaz, C., J. Mieszczanek and M. Bienz (2011). "The Adenomatous polyposis coli tumour suppressor is essential for Axin complex assembly and function and opposes Axin's interaction with Dishevelled." Open Biol **1**(3): 110013.

Moses, K. and G. M. Rubin (1991). "Glass encodes a site-specific DNA-binding protein that is regulated in response to positional signals in the developing Drosophila eye." Genes Dev **5**(4): 583-593.

Perry, M. M. (1968). "Further studies on the development of the eye of Drosophila melanogaster. I. The ommatidia." Journal of morphology **124**(2): 227-247.

Ready, D. F., T. E. Hanson and S. Benzer (1976). "Development of the Drosophila retina, a neurocrystalline lattice." Dev Biol **53**(2): 217-240.

Sprecher, S. G., F. Pichaud and C. Desplan (2007). "Adult and larval photoreceptors use different mechanisms to specify the same Rhodopsin fates." Genes Dev **21**(17): 2182-2195.

Tanaka-Matakatsu, M., T. Uemura, H. Oda, M. Takeichi and S. Hayashi (1996). "Cadherin-mediated cell adhesion and cell motility in Drosophila trachea regulated by the transcription factor Escargot." Development **122**(12): 3697-3705.

Tomlinson, A. (2003). "Patterning the peripheral retina of the fly: decoding a gradient." Dev Cell **5**(5): 799-809.

Treisman, J. E. and G. M. Rubin (1995). "wingless inhibits morphogenetic furrow movement in the Drosophila eye disc." Development **121**(11): 3519-3527.

Waddington, C. and M. M. Perry (1960). "The ultra-structure of the developing eye of Drosophila." Proceedings of the Royal Society of London. Series B, Biological Sciences: 155-178.

Waddington, C. H. and M. M. Perry (1963). "Inter-retinular fibres in the eyes of Drosophila." Journal of Insect Physiology **9**(4): 475-478.

Wang, S. L., C. J. Hawkins, S. J. Yoo, H. A. Muller and B. A. Hay (1999). "The Drosophila caspase inhibitor DIAP1 is essential for cell survival and is negatively regulated by HID." Cell **98**(4): 453-463.

Wehrli, M. and A. Tomlinson (1998). "Independent regulation of anterior/posterior and equatorial/polar polarity in the Drosophila eye; evidence for the involvement of Wnt signaling in the equatorial/polar axis." Development **125**(8): 1421-1432.

Wernet, M. F., T. Labhart, F. Baumann, E. O. Mazzoni, F. Pichaud and C. Desplan (2003). "Homothorax switches function of Drosophila photoreceptors from color to polarized light sensors." Cell **115**(3): 267-279.

Whiteley, M., P. D. Noguchi, S. M. Sensabaugh, W. F. Odenwald and J. A. Kassis (1992). "The Drosophila gene escargot encodes a zinc finger motif found in snail-related genes." Mech Dev **36**(3): 117-127.

Wolff, T. and D. F. Ready (1991). "Cell death in normal and rough eye mutants of Drosophila." Development **113**(3): 825-839.

Xu, C., R. C. Kauffmann, J. Zhang, S. Kladny and R. W. Carthew (2000). "Overlapping activators and repressors delimit transcriptional response to receptor tyrosine kinase signals in the Drosophila eye." Cell **103**(1): 87-97.

Yu, S. Y., S. J. Yoo, L. Yang, C. Zapata, A. Srinivasan, B. A. Hay and N. E. Baker (2002). "A pathway of signals regulating effector and initiator caspases in the developing Drosophila eye." Development **129**(13): 3269-3278.

Zecca, M., K. Basler and G. Struhl (1996). "Direct and long-range action of a wingless morphogen gradient." Cell **87**(5): 833-844.

Zhu, X. D. and P. D. Sadowski (1995). "Cleavage-dependent ligation by the FLP recombinase. Characterization of a mutant FLP protein with an alteration in a catalytic amino acid." J Biol Chem **270**(39): 23044-23054.

4.Discussion

A Wingless gradient emanating from the head capsule leads to the patterning of the retinal periphery. The pigment rim is formed in response to high levels of Wg signaling. In this thesis, I investigated the mechanism by which Wg leads to pigment rim formation. I show that in response to Wg signal activation, there is a developmentally timed sequence of events leading to the apoptosis of the peripheral ommatidia. I also show that the entire eye responds in a manner similar to the periphery upon being subjected to high levels of Wg. While the cone cells are shown to be the primary high threshold responders, there is a logical conundrum in the response of photoreceptors to Wg. Activation of the pathway appears to have no effect on photoreceptor development and survival, while the blocking of Wg transduction prevents the apoptosis of the photoreceptors at the periphery. Lastly, I presented data that suggests that all the cells of the ommatidia respond to the Wg signal in a combinatorial manner to lead to pigment rim formation.

Model for the apoptosis of the peripheral ommatidia

In this thesis, I present evidence to suggest that apoptosis and pigment rim formation at the periphery of the eye results from the concerted response of the cells of the ommatidium. The cone cells act as the 'high threshold' responders to *wg* signaling. Once the threshold is attained, the cone cells express Esg, Wg and Notum. A few hours later, they collapse to the level of the photoreceptor and subsequently undergo apoptosis.

We also show that while the collapse does not preclude the apoptosis of the photoreceptors, it is required for the Wg induced apoptosis of the cone cells. Our

experiments with the activation of Wg signaling in all the cone cells show that although all the cone cells undergo collapse, it is not sufficient to induce their apoptosis, as we see the persistence of the collapsed cone cells in the main body of the eye.

Wg expressed in the cone cells (2° Wg) acts as a 'booster' dose for the remaining cells of the ommatidia. In a wild type retina, only the outermost cone cells express Wg, and the co-expression of Notum possibly restricts the diffusion of this booster dose of Wg, thus only the outermost photoreceptors undergo apoptosis. One hypothesis is that the photoreceptors receive the Wg signal from both the HC and the cone cells in order to undergo apoptosis. This is supported by the observation that there is a broad swathe of peripheral apoptotic ommatidia in *Pros-Gal4; UAS-Arm** eyes. As the cone cells of these eyes all express the 2° Wg, the photoreceptors of the peripheral rows of ommatidia now attain the threshold Wg activation levels and undergo apoptosis. Also, knockdown of the 2° Wg signal by RNAi in *Pros-Gal4; UAS-Arm** eyes shows a wild type pigment rim, suggesting that the additional ommatidial apoptosis did not occur. Pupal development analysis of the eyes of this genotype would provide convincing evidence that the booster dose of Wg is involved in causing the broad swathe of apoptosis. As an alternative approach, we showed that the addition of a tonic dose of Wg signal to the *Pros-Gal4; UAS-Arm** eyes leads to a GMR-wg eye phenotype, lending support to the idea that the 2°Wg itself is the cone-cell derived 'death' signal which leads to photoreceptor apoptosis.

There are two cell types that sense this booster dose of Wg and might be responding to it – the photoreceptors and the surrounding pigment cells. We decided to investigate the

role of photoreceptors first, as it has been widely published that activation of Wg signaling in the photoreceptors causes their apoptosis.

In the experiments designed to understand the role of photoreceptors in the apoptotic cascade, I uncovered a logical conundrum: Blocking Wg signal transduction in the photoreceptors prevents their apoptosis, while activating Wg signal transduction in the photoreceptors alone had no effect on the photoreceptor development. This was a very surprising observation. Having ruled out all the possible technical errors that might lead to this result, one potential explanation is as follows: the photoreceptor apoptosis requires two signaling inputs – they have to receive a high Wg signal during early developmental stages (a priming signal) and a second signal during the pupal stages (a trigger signal). In response to the priming Wg signal, the photoreceptors undergo peripheral specializations, for example DRO formation occurs. This suggests that the photoreceptors can respond to the diffusing Wg signal i.e. there is no block on Wg transduction. This idea is also supported by our observations that blocking Wg transduction in the photoreceptors prevents their apoptosis. Also, in the *Otd-Gal4; UAS-Arm** pupal eyes, we see the transformation of the dorsal half of the retina to DRO (as evidenced by Hth staining); thus indicating that the Wg signaling pathway is activated strongly enough to accomplish the intermediate level molecular response. However, the failure of these photoreceptors to undergo apoptosis subsequently suggests that the trigger signal is missing. As our results indicate that Wg signaling in all the cells of the ommatidia is sufficient for their apoptosis (*GMR-wg* eyes, *GMR-Gal4; UAS-Arm** eyes), the trigger signal for the

photoreceptors must be non-cell autonomous i.e. either the cone cells or the pigment cells must send out a signal in response to strong levels of Wg activation.

An experiment to test this hypothesis is to block the earlier priming signaling and check if the later stage trigger signal is sufficient for apoptosis. This can be done using *Otd-Gal4; UAS-NrtWg; Tubα1-Gal80ts*. Gal80 is a temperature sensitive allele used to suppress Gal4 mediated activation (McGuire, Mao et al. 2004). This allele is active at lower temperatures, and when we shift it to the restrictive temperature, Gal80 is no longer active. The Gal4 is now free to activate transcription of the UAS transgene. The developing larvae are allowed to grow and pupate at 18°C (when Gal80 is active, therefore Wg signal is not activated) until 28hrsAPF, at which point the pupae are shifted to 25°C. Now the photoreceptors express the tethered form of Wg. As we know that *Otd-Gal4; UAS-NrtWg* eyes are smaller in size and sections show *GMR-wg* like tissue, this is a good experimental background to test the presence of a priming signal. If the photoreceptors in these experimental eyes fail to undergo apoptosis, it would suggest that a priming signal is required. It is critical to assess the timed developmental apoptosis in these experiments and compare them with the *GMR-wg* and *Otd-Gal4; UAS-NrtWg* pupal eye development. This is important to distinguish apoptosis of the photoreceptors versus developmental degeneration in response to chronic Wg signaling, as seen in the main body ommatidia of *Pros-Gal4; UAS-Arm** eyes, and in the *Elav(II) Gal4; UAS-Arm** eyes. Alternatively, if the photoreceptors undergo timed apoptosis, it would argue against the priming hypothesis, instead indicating that the levels of Wg signaling activated in *Otd-Gal4; UAS-Arm** are insufficient for the apoptotic response to occur.

The corollary experiment can be conducted using *Otd-Gal4; UAS-AxnGFP; UAS-Gal80*. In this case we are blocking the Wg signal transduction at the later pupal stage in the photoreceptors. If the peripheral photoreceptors in this background undergo apoptosis, it would suggest that as long as the photoreceptors are primed by Wg in the earlier stages, the Wg dependent trigger signal is received from the cone cells or the pigment cells in order for the photoreceptors to die. In case the photoreceptors do not undergo apoptosis, it would suggest that the photoreceptors require Wg signaling in the pupal stages in order to die.

Role of the pigment cells in the *Wg* induced peripheral apoptosis

During formation of the pigment rim, the cells of the outermost ommatidia – the cone cells, photoreceptors and 1°pigment cells undergo apoptosis, while the 2°/3° pigment cells surrounding them coalesce to form the pigment rim. Due to the unavailability of a good pigment cell specific driver line, we were unable to perform direct experiments relating to *Wg* signaling effects in the pigment cells. However, based on a number of observations, we suggest the pigment cells might have a role to play in this Wg dependent apoptotic cascade.

Peripheral gene expression - In response to the Wg diffusing in from the head capsule, Snail class proteins are expressed in the cone cells. Additionally, Esg is also expressed in the 2°/3° pigment cells surrounding the moribund ommatidia. Previous studies indicate that this expression of Esg in the pigment cells is required for the tightening of the lattice into a hexagonal array. Similarly, Notum is also expressed in these pigment cells. This

suggests that the pigment cells also aid in restricting the high level action of Wg to the outermost row of ommatidia.

In the broader swathe of death in the *Pros-Gal4; UAS-Arm** periphery, the booster dose of Wg signal is sensed by the photoreceptors as well as the surrounding pigment cells. We postulate that the pigment cells might be the source of the trigger signal that leads to the photoreceptor apoptosis. The broader region might correspond to the region where the photoreceptors sense the diffusing Wg; additionally they receive the secondary signal from the cone cells and the surrounding pigment cells in order to undergo apoptosis.

APC and Elav (C155) Gal4 experiments - In the D-APC^{Q8} homozygous mutants, as well as in the *Elav (C155) Gal4; UAS-Arm** eyes, we see apoptosis of photoreceptors when there is Wg signaling activation in both photoreceptors and the surrounding pigment cells. The activation of Wg signaling at high levels only in the photoreceptors leads to differentiation and developmental defects as indicated by the *Elav(II)Gal4;UAS-Arm** eye phenotype, suggesting that the pigment cells might be sending out a signal to cause clearance of these ommatidia.

Wg signaling activation in cone cells and photoreceptors - If the pigment cells were not involved in the death cascade, then cell autonomous activation of Wg signaling in the cone cells and photoreceptors should reproduce the *GMR-wg* phenotype. However when we examined the eyes of *ProsGal4; OtdGal4;UAS-Arm**flies, we saw that all the photoreceptors are degenerated and the eye surface lacks lenses. But the remnants of the pigment cell lattice are still present instead of coalescing to form pigment rim like tissue.

It should also be noted that in this experiment, the cone cells should express the 2°Wg signal, thus this booster dose might be involved in the degeneration of the photoreceptors. Since the Wg response in pigment cells might not have been activated at the appropriate stage (32hrsAPF), the pigment cells fail to clear the degenerate optical units. This idea is supported by the fact that when we conducted the same experiment with *Elav (C155) Gal4; ProsGal4; UAS Arm**; all that remained of the eye was a thin strip of pigment cells and bristles, suggesting that there is a requirement for Wg signaling in the pigment cells in order to achieve complete clearance of ommatidia and for the formation of pigment rim.

To better understand the role and timing of response of the pigment cells, we have to follow the pigment cells development in the pupal stages. It would be critical to identify if the primary pigment cells collapse along with the cone cells, and the timing of coalescing of the 2°/3° pigment cells. We have recently acquired a pigment cell Gal4 line (54C), which shows expression in the pigment cells and the central photoreceptors R7/R8. Although it is not a clean driver line, it would be interesting to observe the effects of activating and blocking the Wg signaling pathway in the pigment cells on their own, as well as in conjunction with the other cell type specific driver lines. Most of the studies on pigment cells have focused on their patterning during lattice formation; these experiments would provide information on their signaling properties.

Similar to the pigment rim, a monolayer pigmented epithelium called the Retinal Pigmented Epithelium (RPE) is present in vertebrate eyes as the external layer of the

optic neuro-epithelium, while the inner layer develops as the neural retina (Fuhrmann 2010). It has been reported that activation of BMP and Wnt signaling are required for RPE specification in the chick retina (Steinfeld, Steinfeld et al. 2013). Also, the RPE is involved in maintaining photoreceptor integrity, and is required for the clearance of shed membrane discs and retinoid recycling. Ablation or defects in the RPE can lead to photoreceptor degeneration (Longbottom, Fruttiger et al. 2009, Nasonkin, Merbs et al. 2013). It is therefore possible that the pigment cells in the *Drosophila* retina are also actively involved in the signaling mechanism to lead to Pigment Rim formation. These similarities with the vertebrate eye development also suggest an additional role for the pigment rim in maintaining the stability of the retinal periphery by clearing the degenerate optical units, along with providing optical insulation to the ommatidia.

There are many possible candidates for the pigment cell derived signal: the EGFR pathway is reportedly activated to prevent Wg induced apoptosis, while the N/Dl signaling is actively involved in the pigment cell lattice formation (Freeman and Bienz 2001, Brachmann and Cagan 2003). We investigated the role of blanket expression of these pathways in the eye, but it is not possible to attribute the degeneration of the eyes to the PR forming mechanism versus earlier patterning defects. We have to assess the effects of timed expression of these pathways using the cell type specific driver lines in order to gain information about the signaling by the pigment cells. An alternate possibility is the down regulation of cell-cell contacts by Esg expression in the peripheral pigment cells, which might aid the ommatidial instability and subsequent clearance. As Esg has been reported to be involved in the tightening of the hexagonal lattice in later

stages of pupal development (Lim and Tomlinson 2006), it is possible it plays a similar role in the pigment cells for pigment rim formation.

Another interesting question raised by the work presented in this thesis is: What controls the precise timing of the Wg dependent apoptotic cascade? Although the peripheral ommatidia receive Wg from early on in development, the response is observed only in the mid-pupal stage in a very specific time range. One candidate for the regulation of this timing mechanism is Ecdysone (Ecd) signaling. Ecd is a hormone released from the ring gland in carefully timed bursts that coincide with morphological transitions. The timing of the Wg response at the periphery occurs approximately at the same time as an increase in Ecd signaling. This peak of Ecd might provide the trigger to activate the apoptotic program (Thummel 1996, Thummel 2001). Ecd is also known to be involved in developmental apoptosis in the destruction of larval tissues during metamorphosis, abdominal muscle apoptosis and in salivary gland destruction during the prepupal/pupal transition stages (Zirin, Cheng et al. 2013). A similar role for the Ecd induced timed onset of differentiation has also been reported in the follicle development in ovaries (Bai, Uehara et al. 2000, McDonald, Pinheiro et al. 2003). The role of a strict timing control is also hinted at in our observations of the *Pros-Gal4; UAS-Esg* eyes, where we see precocious stochastic collapse of the cone cells upon ectopic expression of Esg. This suggests that the synchronous response of the entire retina requires an additional signaling input. It would be interesting to test the effects of perturbing Ecd signaling on pigment rim formation, and it might also help explain the unusual role of *wg* as a pro-apoptotic factor in this context.

Bai, J., Y. Uehara and D. J. Montell (2000). "Regulation of invasive cell behavior by taiman, a Drosophila protein related to AIB1, a steroid receptor coactivator amplified in breast cancer." Cell **103**(7): 1047-1058.

Brachmann, C. B. and R. L. Cagan (2003). "Patterning the fly eye: the role of apoptosis." Trends Genet **19**(2): 91-96.

Freeman, M. and M. Bienz (2001). "EGF receptor/Rolled MAP kinase signalling protects cells against activated Armadillo in the Drosophila eye." EMBO Rep **2**(2): 157-162.

Fuhrmann, S. (2010). "Eye morphogenesis and patterning of the optic vesicle." Curr Top Dev Biol **93**: 61-84.

Lim, H. Y. and A. Tomlinson (2006). "Organization of the peripheral fly eye: the roles of Snail family transcription factors in peripheral retinal apoptosis." Development **133**(18): 3529-3537.

Longbottom, R., M. Fruttiger, R. H. Douglas, J. P. Martinez-Barbera, J. Greenwood and S. E. Moss (2009). "Genetic ablation of retinal pigment epithelial cells reveals the adaptive response of the epithelium and impact on photoreceptors." Proc Natl Acad Sci U S A **106**(44): 18728-18733.

McDonald, J. A., E. M. Pinheiro and D. J. Montell (2003). "PVF1, a PDGF/VEGF homolog, is sufficient to guide border cells and interacts genetically with Taiman." Development **130**(15): 3469-3478.

McGuire, S. E., Z. Mao and R. L. Davis (2004). "Spatiotemporal gene expression targeting with the TARGET and gene-switch systems in Drosophila." Sci STKE **2004**(220): pl6.

Nasonkin, I. O., S. L. Merbs, K. Lazo, V. F. Oliver, M. Brooks, K. Patel, R. A. Enke, J. Nellissery, M. Jamrich, Y. Z. Le, K. Bharti, R. N. Fariss, R. A. Rachel, D. J. Zack, E. J. Rodriguez-Boulan and A. Swaroop (2013). "Conditional knockdown of DNA methyltransferase 1 reveals a key role of retinal pigment epithelium integrity in photoreceptor outer segment morphogenesis." Development **140**(6): 1330-1341.

Steinfeld, J., I. Steinfeld, N. Coronato, M. L. Hampel, P. G. Layer, M. Araki and A. Vogel-Hopker (2013). "RPE specification in the chick is mediated by surface ectoderm-derived BMP and Wnt signalling." Development **140**(24): 4959-4969.

Thummel, C. S. (1996). "Flies on steroids--Drosophila metamorphosis and the mechanisms of steroid hormone action." Trends Genet **12**(8): 306-310.

Thummel, C. S. (2001). "Molecular mechanisms of developmental timing in C. elegans and Drosophila." Dev Cell **1**(4): 453-465.

Zirin, J., D. Cheng, N. Dhanyasi, J. Cho, J. M. Dura, K. Vijayraghavan and N. Perrimon (2013). "Ecdysone signaling at metamorphosis triggers apoptosis of Drosophila abdominal muscles." Dev Biol **383**(2): 275-284.

Appendix 1: Genetic screen to identify modifiers of the Wingless signaling pathway

Introduction

As described in detail in the Section 1, the presumptive head capsule (HC) tissue surrounding the developing retina of *Drosophila* expresses Wg (Ma and Moses 1995, Treisman and Rubin 1995), and this diffusing Wg forms a gradient at the periphery of the pupal eye (Wehrli and Tomlinson 1998, Tomlinson 2003). This gradient is interpreted to form the peripheral retinal specializations : low levels of Wg prevent bristle formation (Cadigan, Jou et al. 2002); intermediate levels of Wg lead to formation of plane polarized light detectors, called the Dorsal Rim Ommatidia (DRO) (Wernet, Labhart et al. 2003); and high levels of Wg lead to the formation of the pigment rim (Tomlinson 2003). Wg signaling from the surrounding HC induces its own expression in the cone cells of the peripheral ommatidia at 32hrsAPF. It was also shown that the cone cells of these peripheral ommatidia express the Snail class family of zinc-finger transcription factors (Escargot, Snail and Worniu) and Notum, an α/β hydrolase that acts to restrict Wg diffusion. Furthermore, it was shown that these responses were required for the peripheral ommatidial apoptosis (Lim and Tomlinson 2006).

Based on these observations, we wished to identify novel genes that might be involved in the formation of the pigment rim. To do so, we decided to conduct a genetic screen for novel modifiers of the peripheral patterning process.

As shown in Section 3: Results – part 1.2, ectopic expression of Wg in all the cells of the ommatidia using *GMR-wg* transgene leads to the formation of a pigment-rim like 'pseudo-periphery', which we then utilized to understand the mechanisms underlying

pigment rim formation. We also showed that cell autonomous activation of the Wg pathway in all the cells of the eye, using an activated form of Armadillo (the fly β-catenin) (*UAS-Arm*; GMR-Gal4*) (Results chapter, Figure 11A –D) leads to a similar phenotype, albeit slightly milder than a *GMR-wg* eye. As the *UAS-Arm*; GMR-Gal4* phenotype is milder, it provides us with a sensitized background against which we can screen for modifiers (enhancers and suppressors) of the peripheral patterning process.

Given that the cone cell response involves the expression of the Snail family of transcription factors, we also wished to identify genes that might be activated downstream of these transcription factors. The modifiers identified in the latter screen were expected to be a subset of the modifiers identified in the *UAS-Arm*; GMR-Gal4* screen, thus helping to establish a potential network of genes that are involved in pigment rim formation.

In response to Wg signaling, only the cone cells of the peripheral ommatidia express Esg. Thus the ideal screening background for downstream signaling modifiers would have been *UAS-Esg; Pros-Gal4* eyes, wherein all the cone cells of the retina are expressing Esg. Unfortunately, these flies are sterile and unviable. Therefore we decided to screen *UAS-Esg; GMR-Gal4* flies for potential modifiers acting downstream of Esg. These flies show slightly smaller eyes than wild type flies, and the eyes are rough in appearance.

F1 genetic screen experimental design

We wished to identify novel target genes that act downstream of Wg and/or Esg to generate the pigment rim, so we conducted a genetic screen using two strategies –

knockdown of target genes via RNAi, and haplo-insufficiency screen using deficiency lines. Using this approach, we wished to identify genes that modified the sensitized screening backgrounds.

We classified the modifiers as either suppressors i.e. they transformed the eye to a more wild type appearance, or enhancers of the phenotype i.e. they led to further reduction in eye size and more degeneration of the retinal tissue. The cross strategy is as follows:

UAS-dicer; GMR-Gal4; UAS-Esg X UAS-RNAi lines

*UAS-dicer; GMR-Gal4; UAS-Arm** X UAS-RNAi lines

GMR-Gal4; UAS-Esg X Deficiency bearing lines

*GMR-Gal4; UAS-Arm** X Deficiency bearing lines

From the screen, we expected some modifications to be most likely due to reduction in function genes that are already known to be involved downstream in the Wg signaling cascade. These candidate modifiers would validate our screening strategy. Besides these targets, we screened for any novel targets that might play a role in peripheral patterning.

Materials and Methods

All the screen crosses were performed in triplicate at 25°C under standard conditions. The RNAi lines for screening were from VDRC, while the Deficiency lines kit was a kind gift from B. McCabe.

Results

Deficiency screens

As mentioned above, we performed a screen with the available deficiency lines. For some closely-linked, redundant genes, knockdown of one gene is often insufficient to produce a phenotype. However, with our sensitized screening background, we can easily pick up modifications due to 50% reduction in gene dosage of the entire deficiency region. The idea here is to identify regions that modify the pseudo-periphery phenotype upon reduction in gene dosage.

Out of 250 deficiency lines screened, we obtained 20 modifier deficiencies for the *GMR-Gal4; UAS-Arm** phenotype, of which 15 showed mild enhancement of the phenotype, as judged by reduction of external eye size and 5 showed partial suppression, as judged by the relative increase in the eye size. Upon further analyzing these regions with available deficiency lines bearing smaller, overlapping deficiencies, we could not detect significant modification of the *GMR-Gal4; UAS-Arm** phenotype, hence we did not pursue this line of thought any further.

We did not find any significant modifiers of the *GMR-Gal4; UAS-Esg* phenotype in our haplo-insufficiency screen.

RNAi screen

We screened 460 UAS-RNAi lines from the Vienna collection of UAS-RNAi lines.

The control crosses for the RNAi screen were *UAS-dicer; GMR-Gal4* X UAS-RNAi lines. The progeny from these crosses were analyzed for modifications at the periphery,

as well as for effects in the main body of the eye that are independent of our screen phenotype.

Out of all the lines screened, we obtained 14 modifier lines, consisting of 4 suppressors and 10 enhancers of the *GMR-Gal4; UAS-Arm** phenotype.

Gene name	**Modification of *GMR-Gal4; UAS-Arm**** **phenotype**	**Control**
optomotor-blind related gene-1	Enhancer	No effect
H15	Enhancer	Enhancer
tis 11 homolog	Enhancer	Enhancer
vielfaltig/zelda	Enhancer	Enhancer
not1	Enhancer	Enhancer
tachykinin-like receptor at 99D	Mild enhancer	No effect
pangolin	Suppressor	No effect
CG1764	No change	No effect
H6-like-homeobox	Enhancer	Enhancer

Homeodomain protein 2.0	Enhancer	Enhancer
taiman	Enhancer	Enhancer
ζ trypsin	Enhancer	Enhancer
tramtrack	Enhancer	Enhancer
CG18367	Enhancer	Enhancer

Note: Enhancement in control indicates a rough eye as compared to a WT eye.

Of the 460 RNAi lines screened, we obtained 21 modifier lines, all of which were enhancers of the *GMR-Gal4; UAS-Esg* phenotype.

Gene name	**Modification of *GMR-Gal4; UAS-Esg* phenotype**	**Control**
sine oculis	Enhancer	Enhancer
dmyc	Enhancer	Enhancer
combgap	Enhancer	Enhancer

caupolican	Enhancer	No effect
CG2116	Enhancer	No effect
CG12370	Enhancer	No effect
CG15440	Enhancer	No effect
DNA polymerase α60kD	Enhancer	No effect
split ends	Enhancer	Enhancer
cyclin J	Enhancer	No effect
pointed	Enhancer	Enhancer
hr4	Enhancer	Enhancer
odd skipped	Enhancer	Enhancer
H6-like-homeobox	Enhancer	Enhancer
Homeodomain protein 2.0	Enhancer	Enhancer
taiman	Enhancer	Enhancer
ζ trypsin	Enhancer	Enhancer
tramtrack	Enhancer	Enhancer

H15	Enhancer	Enhancer
CG18367	Enhancer	Enhancer

Note: Enhancement in control indicates a rough eye as compared to the WT eye.

From both these screens, we obtained seven common modifiers.

We reconfirmed these results using multiple independent transgenic RNAi lines (targeting different regions of the transcripts) against these genes available from the VDRC. All lines tested gave similar result, except for 2 suppressors of GMR-Arm*. After reconfirmation of these results, we shortlisted 25 modifiers for further analysis.

For further analysis of these modifiers, we used the following criteria:

- Knockdown on its own causes a peripheral eye phenotype.
- Checked UAS lines for rescue of the peripheral ommatidial death, and of the *GMR-wg* induced pseudo periphery.
- Checked effects of knockdown in the wing using a wing-specific driver line MS1096, to assess if we were simply picking up generic cytotoxic modifiers or if the effect was specific to the eye.
- Checked expression of available reporters and antibodies to see expression in retina at the correct developmental stages.

Based on these experiments, we found two modifiers that showed interesting peripheral patterning phenotypes in the eye:

1. *taiman (tai)* – It is an Ecdysone receptor cofactor (Bai, Uehara et al. 2000), and its knockdown in the eye (*GMR-Gal4; UAS-tai-RNAi*) causes early pupal lethality. Ectopic expression with *UAS-tai; GMR-Gal4* partially rescued the *GMR-wg* pseudo periphery eye phenotype. Remnants of ommatidial structure and a few photoreceptors survive. On its own, we see a definite survival of some peripheral ommatidia in *GMR-Gal4; UAS-tai* eyes (Figure 1A). Furthermore, there is some reduction in the number of Dorsal Rim Ommatidia (DRO), and there are very few bristles. These observations indicated that Tai might be involved in the Wg signal transduction pathway at the periphery of the eye.

   Upon checking the developmental stages, we see that there is no obvious reduction in the Hth and Wg expression regions (Figure1 B,C). Apoptosis occurs at the appropriate time in the periphery. Upon testing the various pupal developmental stages of *GMR-Gal4; UAS-tai* retinas, we conclude that there is no modification in the peripheral apoptotic cascade. There is a lack of bristle specification; however, that doesn't directly relate to my project, so we did not pursue this analysis any further.

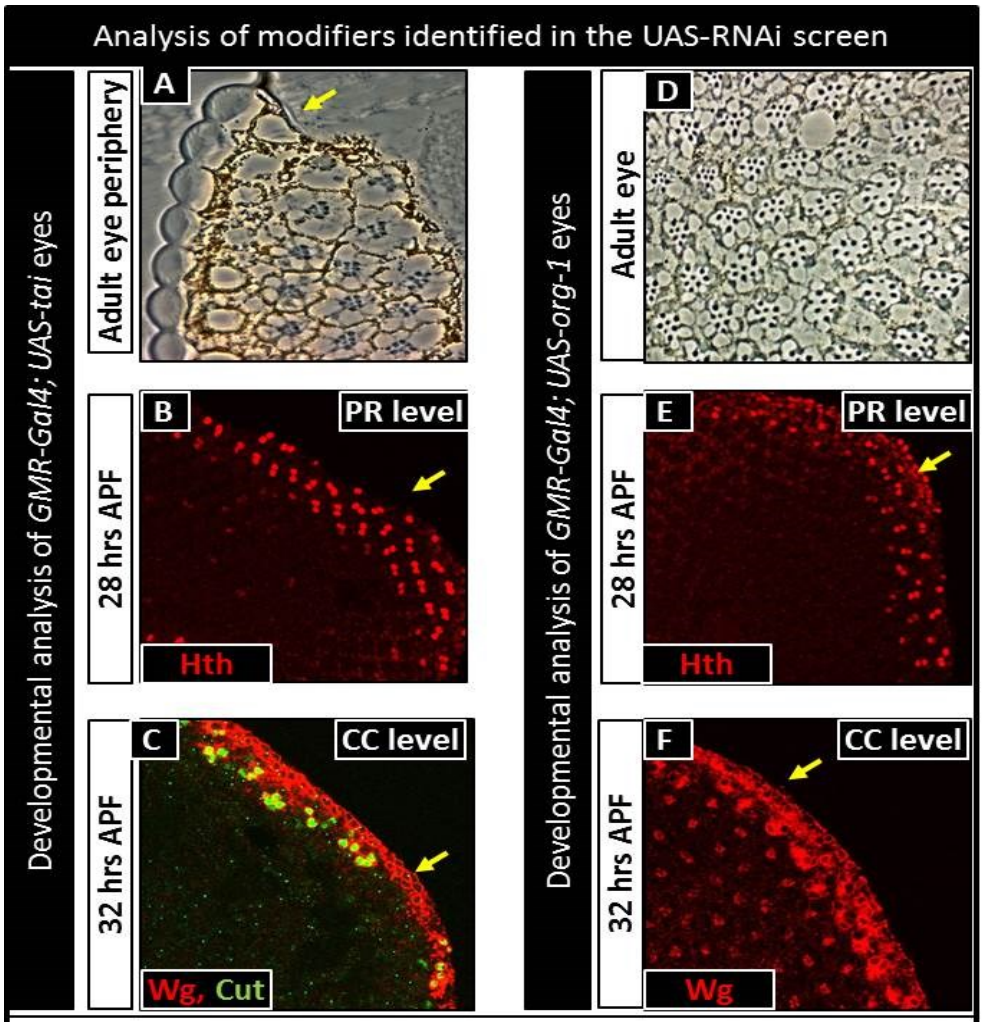

Figure 1: Over-expression of modifier genes in the eye to assess their effects on peripheral patterning.
Note: Yellow arrow indicates the HC.
A-C: *GMR-Gal4; UAS-tai* eyes.
A: The adult eye periphery shows the presence of a few ommatidia adjacent to the HC.
B: Wild type expression of Hth at the periphery.
C: Wild type expression of Wg in the peripheral cone cells.
D-F: *GMR-Gal4; UAS-org-1* eyes.
D: Adult eye sections show slightly disorganized ommatidial array.
E: Wild type expression of Hth at the periphery.
F: Wild type expression of Wg in the peripheral cone cells.

2. *omb-related-gene-1 (Org-1)* – It is a T-box transcription factor (Schaub, Nagaso et al. 2012) that caused enhancement of the *GMR-Gal4; UAS-Arm** eye phenotype upon knockdown via RNAi. *GMR-Gal4; UAS-org1* has a balding phenotype, the entire eye lacks bristles, suggesting that it might be acting in the Wg induced eye patterning process. The adult eye sections show a slightly disorganized pigment cell lattice (Figure 1D). However, it does not rescue the *GMR-wg* induced pseudo periphery phenotype in the eye. Upon examining the pupal development stages of *GMR-Gal4; UAS-org1* eyes, we see that Hth and Wg are expressed in the WT pattern (Figure 1 E, F), and the peripheral development occurs normally. Based on these observations, we did not pursue this analysis any further.

Some of the other modifier genes analyzed *(H15, Tis-11 homolog* and *split-ends*) showed interesting eye development defects, however as they were not related to our project aims, we did not analyze them further.

Discussion and future directions

A genetic screen is a powerful tool to identify novel genes in any sensitized background, however the subsequent unmasking of the relevant genes is dependent on two things: the question we seek to address with the screen, and the analysis of the data obtained. Our screens did not yield much information by way of furthering our knowledge about the Wg induced retinal apoptosis during mid-pupal development. The various reasons for the same are discussed below:

- We used *GMR-Gal4* as a blanket driver line to test the effect of gene knockdown in the entire retina. This was unavoidable at the time as the *Pros-Gal4; UAS-Esg* flies are sterile, and we wished to ascertain if there were downstream modifiers besides the Snail family transcription factors. But since *GMR-Gal4* is expressed from the third larval instar onwards and throughout pupal development, it is difficult to analyze if the gene knockdown causes early specification defects therefore leading to a rough eye or if it is involved in pupal stage apoptosis. One way to circumvent this caveat is to analyze the developmental stage at which the genes affect eye development, but this makes the entire screening process too cumbersome and time consuming, therefore rendering it implausible.

- One of the modifiers we obtained was Pangolin – the fly TCF. This validated our screen strategy as anticipated. Some of the other modifiers were parts of signaling cascades already known to play a role in eye development, for e.g. *pointed* and *tramtrack* are both part of the EGFR signaling pathway. *sine oculis* is an early retinal determination gene. Since we screened for genes that modified the external retinal appearance of the eyes in the control and experimental genotypes, we might have picked up many more generic eye fate determinants or cellular survival genes as opposed to genes specifically involved in the mid-pupal apoptosis.

- The apoptosis phenotype we are investigating involves the death of different cell sub types in response to the external Wg signal. So far we have knowledge of the transcriptional response of one subtype, namely the cone cells. If there are genes that are playing a role in this death cascade by being specifically up regulated in a subset of the cells of the ommatidia, then our screen strategy might not have picked up those modifiers.

- For the modifiers we found, most of them were just annotated CG numbers, with no information or tools available to analyze the gene products. Generation of the tools required to assess the potential role of these genes was time consuming and yet not very informative.

- The subset of available lines that we screened was by no means exhaustive. Hence it is likely that we might find more suitable candidates if were to conduct a more thorough screen of all the available lines.

In order to address these caveats, we need to design the screen background with the temporal controls in mind. Adding a Gal80ts suppressor to the experimental background would enable us to fine tune the timing of expression of the UAS transgene, therefore allowing the eye to undergo normal early differentiation and cause gene knockdown in later pupal stages. Another alternative is to utilize the now characterized cell specific driver lines (*Pros-Gal4* and *Otd-Gal4* for cone cells and photoreceptors respectively) and then analyze the rescue of *GMR-Gal4; UAS-Arm** or *GMR-wg* eye phenotype, along with the Gal80 transgene for

temporal control. Another viable strategy to test for any potential 'death signal' emanating from the cone cells, is to utilize *Pros-Gal4; UAS-Arm** flies as the sensitized screening background. As explained in the main body of the thesis, these flies display an eye phenotype approaching *GMR-wg* pseudo-periphery, but incompletely so. Analyzing the cone cell specific knockdown of genes in this background might help us identify any potential genes involved in signaling between the cone cells and the photoreceptors, thereby enabling a concerted collapse of the ommatidium as a whole.

Bai, J., Y. Uehara and D. J. Montell (2000). "Regulation of invasive cell behavior by taiman, a Drosophila protein related to AIB1, a steroid receptor coactivator amplified in breast cancer." Cell **103**(7): 1047-1058.

Cadigan, K. M., A. D. Jou and R. Nusse (2002). "Wingless blocks bristle formation and morphogenetic furrow progression in the eye through repression of Daughterless." Development **129**(14): 3393-3402.

Lim, H. Y. and A. Tomlinson (2006). "Organization of the peripheral fly eye: the roles of Snail family transcription factors in peripheral retinal apoptosis." Development **133**(18): 3529-3537.

Ma, C. and K. Moses (1995). "Wingless and patched are negative regulators of the morphogenetic furrow and can affect tissue polarity in the developing Drosophila compound eye." Development **121**(8): 2279-2289.

Schaub, C., H. Nagaso, H. Jin and M. Frasch (2012). "Org-1, the Drosophila ortholog of Tbx1, is a direct activator of known identity genes during muscle specification." Development **139**(5): 1001-1012.

Tomlinson, A. (2003). "Patterning the peripheral retina of the fly: decoding a gradient." Dev Cell **5**(5): 799-809.

Treisman, J. E. and G. M. Rubin (1995). "wingless inhibits morphogenetic furrow movement in the Drosophila eye disc." Development **121**(11): 3519-3527.

Wehrli, M. and A. Tomlinson (1998). "Independent regulation of anterior/posterior and equatorial/polar polarity in the Drosophila eye; evidence for the involvement of Wnt signaling in the equatorial/polar axis." Development **125**(8): 1421-1432.

Wernet, M. F., T. Labhart, F. Baumann, E. O. Mazzoni, F. Pichaud and C. Desplan (2003). "Homothorax switches function of Drosophila photoreceptors from color to polarized light sensors." Cell **115**(3): 267-279.