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Cell Cycle Regulation and Pattern Formation in the *Drosophila* Compound Eye: Role of the *retina aberrant in pattern* (*rap*) Gene.

By

Angel C. Pimentel

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirement for the degree of Doctor of Philosophy, The City University of New

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ABSTRACT

Cell Cycle Regulation and Pattern Formation in the *Drosophila* Compound Eye: Role of the *retina aberrant in pattern* (*rap*) Gene.

By

Angel C. Pimentel

Adviser: Dr. Tadmiri Venkatesh

During the development of multicellular organisms, cell proliferation, cell growth and cell death are coordinated with cell fate specification and pattern formation. The timely exit of precursor cells from mitotic cell cycles is critical for proper development and pattern formation. The mechanisms that orchestrate the link between mitotic cell cycles and pattern formation are not well understood. In this dissertation I have used the development of the *Drosophila* compound eye as an experimental system to understand these mechanisms. My studies have focused on the *rap (retina aberrant in pattern)* gene and its role in the development of the *Drosophila* compound eye. Analysis of the cellular pattern formation in *rap* loss-of-function mutants showed that in *rap* mutants photoreceptor neurons R1, R6 and R7 fail to differentiate. In addition, *rap* mutants have aberrant numbers of cone and pigment cells leading to altered retinal patterning. Examination of the mitotic pattern in the developing eye disc revealed that, in *rap* mutants, precursor cells failed to arrest at the G1 stage and underwent abnormal

additional mitotic cycles. Some of the extra cells generated were eliminated by apoptosis. Molecular cloning and DNA sequence analyses revealed that *rap* encodes Fizzy-related (Fzr), a protein with WD (Trp-Asp) repeat domains. Rap/Fzr is a component of the Anaphase Promoting Complex (APC), a multi-protein ubiquitination complex involved in the timely degradation of mitotic cyclins. Results from the Rap/fzr expression studies are consistent with its role in cell cycle exit prior to cell fate specification. Loss-of-function mutations in *rap/fzr* show abnormal accumulation of cyclin B in the developing eye leading to additional mitotic cycles. Targeted overexpression of Rap/Fzr in the developing eye primordia using the *GAL4-UAS* system resulted in premature mitotic exit and either a drastic reduction or elimination of the eye. However, precocious mitotic exit did not inhibit neural differentiation. Differentiated neurons underwent endoreplication cycles giving rise to abnormally large cells and ectopic tumors. Interestingly, targeted expression of Rap/Fzr also resulted in the induction of ectopic antenna. These results suggest that Rap/Fzr plays a key role in the events leading to cell cycle exit and neuronal patterning in the developing eye.

Acknowledgments

The long journey to a Ph.D. degree did not start when I register in the program seven years ago. Rather, It began the day I was born. Thus, I must first thank my parents Julio Ramon Pimentel Rodriguez and Carmen Crespo del Valle de Pimentel. From my father I learned that life was a constant fight, a fight that does not end until the very end. He fought until the end. I miss his voice every Saturday. My mother taught me that if I were consistent and persevere in acquiring my goals, I would finally achieve them. I still can smell the coffee that she prepared for me every morning before school. I am very grateful to my wife, Melania Mercado-Pimentel for her patience and understanding during all these years. A lot of credit should be given to my daughter, Bianca Pimentel Mercado, because she has been part of this voyage since day one. I hope that all the sacrifices served not only as an example but also as a lesson, that in life you have to work hard to succeed. Bianca taught me how simple life is and that the little things in life are the ones that remain in our memories forever. I want to express my gratitude to Richard Sorrentino, Krishnan Sundar and Vanneta Hyatt for proof reading my thesis, to Jorge Morales in the Biology Department for his technical help in the preparation of the figures and with the scanning electron microscope, to Roberto del Valle for helping with the plastic sections of the tumors. I also want to thank Hoda Shamloula and Mkajuma Mbogho for sharing and listening my disappointments when experiments went wrong. Thanks to Dr. Mike Fishman who recruited me from Puerto Rico and never let me down. Thanks to the Faculty of the Biology Department at Cayey University College for cultivating my love for science and for encouraging me to continue graduate studies.

Sometimes life gives you the chance to meet special human beings. Life gave me the opportunity to meet Dr. Tadmiri Venkatesh (Venky). Getting my Ph.D. would not be possible without the help, advice and support of Venky. I first started to work with Venky as his technician. I had already gave up the idea of continuing my graduate studies when we first met. However, he turned my discouragement into motivation. He took my hand and guided me step by step on how to become a good scientist. More than my advisor, he has become a father figure in my life. I will always be grateful to the help and support that Venky gave me during all these years.

I shall not be afraid of storms, cause I have learned to sail my ship.

(Quotation posted at Saint Luke's Episcopal Church
141st and Convent Ave. Harlem)

Dedicatory

I would like to dedicate this thesis to God for giving me the opportunity to live, to my parents Carmen del Valle de Pimentel and Julio R. Pimentel for their guidance in my life, to my wife Melania Mercado-Pimentel for her love and understanding and to the light of my eyes, Bianca Eileen Pimentel for showing me how beautiful and simple life is.

... as in the beginning of times life is full of challenges. I just finish this one. I am ready for the next....

Angel C. Pimentel

Table of Contents

List of Tables

Table Page

Chapter I

Chapter II

List of Figures

Chapter III

Chapter I

Introduction

A. Cell Cycle Regulation and Pattern formation.

In multicellular organisms, cellular pattern formation is coordinated with mechanisms that regulate cell proliferation, growth and death. During development cells continuously go through mitotic cell cycles until the correct number of precursor cells are generated and then, exit the cell cycle. The timing of cell cycle exit frequently coincides with the onset of the expression of genes that define developmental fate (Edlund and Jessell, 1999). Thus, cells must possess an internal mechanism regulating their timely exit from cell cycle and the same mechanism could contribute to the specification of cell fates. Cell cycle is driven by cell dependent kinase proteins (Cdks) with their specific cyclin activators. Cdks are regulated by proteins called cyclins which are required for the kinase activity of the Cdks as well as for the substrate specificity of the Cyclin/Cdk complex (Koepp et al., 1999).

Neuronal differentiation is particularly interesting because once the neurons differentiate they lose the ability to re-enter the cell cycle. The final cell cycle division in many vertebrate neural cells is followed by a dynamic change in the expression of transcriptional regulators, which suggests that the expression of these proteins may signal the cell to leave the cell cycle and also participate in the mainteinance of the post mitotic state of neural progenitor cells (van Lookeren Campagne and Gill, 1998). For example, during neurogenesis in vertebrates and in *Drosophila* the basic helix-loop-helix (bHLH) factors are activated. These factors contribute to the determination of neuronal fate and

later in development they promote different aspect of neuronal differentiation (Lee, 1997). Specifically, in *Xenopus* embryos a subset of these bHLH proteins, neurogenins 1 and 2, as well as NeuroD, are sufficient to promote the ectopic expression in ectodermal cells of markers characteristic of postmitotic neurons (Lee et al., 1995; Ma et al., 1996; Olson et al., 1998). Therefore, it is possible that neurogenic bHLH factors may interact with the core cell cycle machinery through the induction of expression or activation of Cdk inhibitors.

It has been proposed that cell cycle exit determines the timing of action of transcription factors that specify neuronal subtype identity (Edlund and Jessell, 1999). Studies of motor neuron differentiation support this idea (Tanabe et al., 1998). When misexpressed in the neural tube cells, the MNR2 homeodomain protein is able to initiate a program of somatic motor neuron differentiation, such as the expression of homeodomain proteins, neurotransmitter phenotype and axonal trajectory (Tanabe et al., 1998). However, it appears that the induction that MNR2 triggers is operated by the regulation of an independent developmental program that controls the time at which neural progenitor cells exit the cell cycle (Tanabe et al., 1998). Downstream genetic targets of MNR2 that are normally expressed in postmitotic motor neurons can be ectopically induced by MNR2 only after progenitor cells have left the cell cycle (Tanabe et al., 1998).

Another possibility is that once progenitor neural cells exit the cell cycle, they will lose their ability to further respond to extrinsic signals (Edlund and Jessell, 1999). In this scenario, the fate of the progenitor cells is determined by the intrinsic determinants that they express at the moment at which the cells leaves the cell cycle. These

determinants are established as a response to the environmental signals that the cells are exposed to during their final cell division cycle (Edlund and Jessell, 1999). The vertebrate central nervous system shows some examples in which the specific type of neuron that develops will depend on the time at which cells exit the cell cycle (Edlund and Jessell, 1999). One such example is the cerebral cortex. Neural progenitors of this region that leave the cell cycle precociously will acquire neuronal identities different from those neural progenitors which leave the cycle at later times (Frantz and McConnell, 1996; McConnell, 1995). A similar situation is found in the progenitor cells of the spinal motor neuron within the medial and lateral division of the lateral motor column (Hollyday, 1983). Therefore, the differentiation of the pluripotent neural progenitor cells into specific neurons seems to be related to the cell cycle events that precede such differentiation (Edlund and Jessell, 1999). In some systems the commitment of certain neurons appears to be restricted several divisions before the cell cycle exit. For others neurons similar restriction appears to occur much closer to the time of the cell cycle exit, whereas for a third class of neurons the restriction appears only after the cell cycle exit (Edlund and Jessell, 1999).

Studies of oligodendrocyte differentiation have also suggested that an internal cellular mechanism regulates the timely exit of the cell cycle (Raff et al., 1998). The proliferation of oligodendrocytes depends on the expression of mitogenic factors. However, even if the cells are saturated with mitogenic factors they will eventually exit the cell cycle and, in the presence of appropriate hormones, they will differentiate into oligodendrocytes. In vitro studies of these cells have revealed that the clonal progeny of an individual oligodendrocyte progenitor cells will differentiate in near synchrony. These

observations suggest that an intrinsic mechanism regulates the timing of cell cycle exit, as well as cell differentiation (Raff et al., 1998; Temple and Raff, 1986).

An important issue that my dissertation deals with is the contribution of cell cycle exit to the neural cell differentiation. The developing eye disc offers an attractive system to study cell cycle regulation and its relationship to the specification and patterning of neuronal cells. In the developing *Drosophila* eye, mitotic cell cycle is precisely coordinated with a continuous temporal gradient of different molecular cues necessary for the proper differentiation of the ommatidium. Thus, the relationship of these intrinsic mechanisms of neural differentiation signals and the cell cycle exit can be easily studied in this system.

B. Structure and Organization of the Compound Eye.

The *Drosophila* visual system consists of the compound eye, the optic ganglia and the region of the central nervous system that processes the information of the visual system (Meinertzhagen, 1973). The compound eye of *Drosophila* is comprised of about 800 repetitive units called ommatidia (Ready, 1989). These units are arranged in a very precise manner giving the eye a smooth external appearance (Figure 1). Each ommatidium contains invariable number of cells; eight photoreceptor cells, 4 cone cells that secrete the lens, 6 pigment cells, that optically isolate ommatidia from one another, and four cells of the mechanosensory bristle complex (Figure 2). The plasma membrane of the photoreceptor cells (R-cells) has multiply folds, which create microvillar membrane stacks, called rhabdomeres. Rhabdomere from each photoreceptor cell extend into the central space of the ommatidium. The rhabdomeres contain the photopigment

rhodopsin (Smith et al., 1991). The R-cells can be classified into three subtypes: R1-R6, R7 and R8 based on their position within the ommatidia, the type of opsin they express, and their synaptic connectivity ganglia (Table 1). The first subtype consists of photoreceptors R1-R6. They are found in the periphery of the ommatidium, they contain opsin Rh1 which is sensitive to the green spectrum of light and they synapse in the lamina (Braitenberg, 1967; Meinertzhagen, 1973; Strausfeld, 1976). The second subtype consists of photoreceptor R7. R7 cell expresses opsins Rh3 or Rh4, which absorbs in the UV wavelength of light. The Rh3 and Rh4 opsins are expressed in two mutually exclusive subsets of R7-cells (Feiler et al., 1992; Fortini and Rubin, 1990). The R7 cells are located centrally within the ommatidia, in the same plane but distal to the R8 cell. The axons of photoreceptor R7 synapse in the medulla, the second optic ganglion. The third subtype consists of photoreceptor R8. It is found in a central position within the ommatidia, basal to R7. It expresses the opsin Rh5 and Rh6 in non-overlapping subset of cells (Chou et al., 1999; Salcedo et al., 1999). The axons of R8 synapse in the medulla. The rhabdomeres of R1-R6 extend the entire length of the retina, whereas, rhabdomeres of R7 and R8 are shorter in length and are smaller in profile (Braitenberg, 1967).

Figure 1. Structure and organization of the *Drosophila* compound eye. A) Scanning electron micrograph of the compound eye shows a smooth external surface. B) A section tangential to the surface of the eye reveals the reiterated pattern of ommatidial organization.

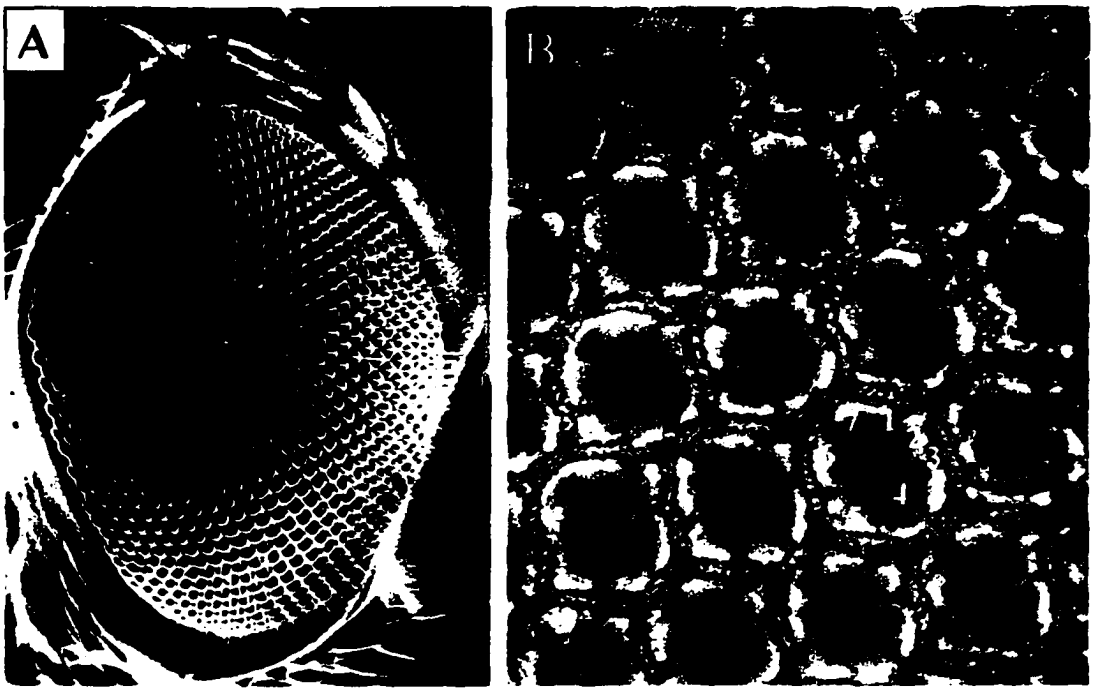


Figure 2. Graphical representation of a single ommatidia. Each ommatidium is comprised of eight photoreceptor cells and eleven accessory cells. The cell membrane of each photoreceptor cell is multiply folded creating a microvillar membrane stacks, called rhabdomere. The rhabdomeres contain the photoreceptor pigment rhodopsin and they extend to the center of the ommatidia. The rhabdomeres of the photoreceptors R1-R6 are larger in profile; they occupy the periphery of the cluster and extend lengthwise ommatidia. The rhabdomeres of R7 and R8 cells are smaller in profile they occupy the center of the cluster and extend partially the length of the ommatidia. The R7 cell is on top of R8 cell, which is basal in position in the ommatidia. Four cone cells are found overlying the photoreceptor cells. The surface of the pseudocone is covered with small papillae from which lens material is secreted (Wolff and Ready, 1993). Each cone cell extends a thin process between the photoreceptor cells up to the base of the ommatidium where it plugs the ommatidial cavity (Wolff and Ready, 1993). The rhabdomeres of the photoreceptor cells end just above this plug. The axons from the photoreceptor neurons begin immediately bellow this plug (Wolff and Ready, 1993). The cone cells in conjunction with the primary pigment cells create the corneal lens. This lens is a chitinous extracellular secretion produced by these cells. The pseudocone is a secondary lens element. As with the corneal lens, the pseudocone is created by an extracellular secretion of the cone cells and primary pigment cells. However, this secretion is different from the cornea lens because it is non-chitinous. The pseudocone is made in later pupal life, after and under the corneal lens (Wolff and Ready, 1993). Cone cells are surrounded by two primary pigment cells. These cells form the inner layer of the pseudocone chamber, and are the only cells of the eye that do not reach the floor of the ommatidial (Wolff and Ready, 1993). The secondary and tertiary pigment cells surround each other ommatidia imposing the order in the ommatidial array (Wolff and Ready, 1993). They contain pteridine (red screening pigment) as well as ommochrome pigment granules (Wolff and Ready, 1993). The retina is supported by a specialized basement membrane known as the fenestrated membrane, which is composed of the flattened feet of the secondary and tertiary cells with a thin basal lamina (Wolff and Ready, 1993). The mechanosensory bristle of the eye is constructed in late pupal life. They are developmentally different from the ommatidia and it projects its axon to a different region called dorsal deuterocerebrum. (Figure and legend were reprinted from (Wolff and Ready, 1993) with permission from CSHL Press).

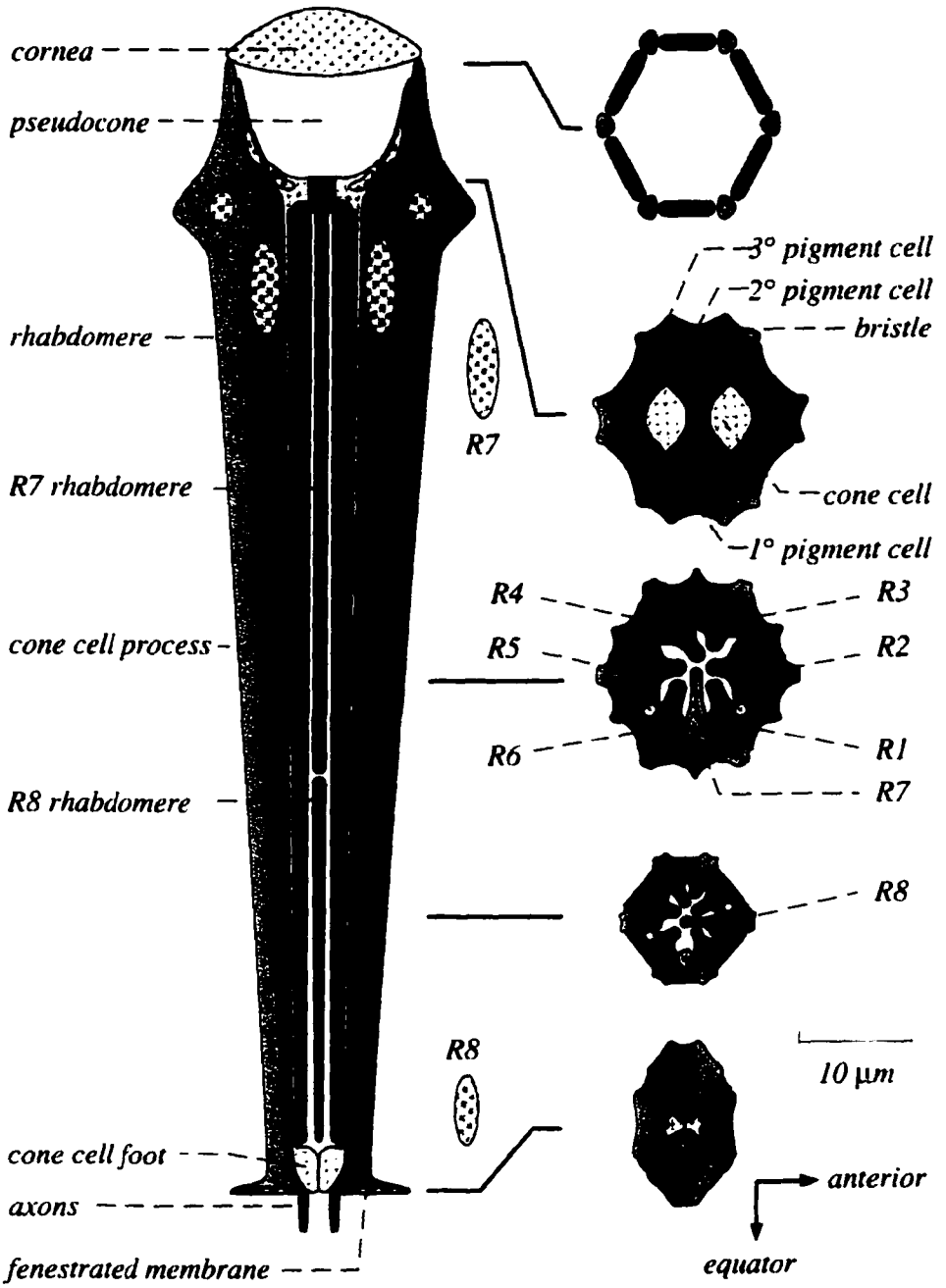


Figure 2 (See facing page for legend.)

Figure 2

PHOTORECEPTOR SUBTYPES	SYNAPTIC CONNECTIVITY	OPSIN TYPE	EXCITATION WAVELENGTH	POSITION WITHIN THE CLUSTER
R1-R6	Lamina	Rh1	Green	Periphery
R7	Medulla	Rh3 & Rh4	UV	Center
R8	Medulla	Rh5 & Rh6	Blue & Green	Center

Table 1. Photoreceptor (R cells) subtypes, opsins and connectivity.

Development of the Compound Eye.

The development of the *Drosophila* compound eye begins at the syncytial blastoderm stage during which approximately 20 progenitors cells are set apart (Wieschaus and Gehring, 1976). This group of cells starts to proliferate in the late embryo and continues until the second larval instar, forming a monolayer of undifferentiated epithelial cells known as the eye-antennal imaginal disc (Garcia-Bellido and Merriam, 1969; White and Kankel, 1978). Differentiation of the eye disc begins during third instar with the appearance of a dorsal-ventral groove in the undifferentiated epithelium. This groove is known as the morphogenetic furrow (MF) and is the result of a coordinated basal migration of nuclei of the cells during the beginning of the ommatidial differentiation. (Cagan, 1993; Ready et al., 1976). The MF is initiated at the posterior end of the eye disc and as it moves anteriorly, the monolayer of cells is transformed into a precise three-dimensional array of photoreceptor and accessories cells (Wolff and Ready, 1991). While these events are occurring, cells ahead of the furrow remain undifferentiated. The ommatidial photoreceptor clusters are specified one column at a time, beginning in the posterior part of the eye disc. The columns of photoreceptor clusters emerge from the morphogenetic furrow at a rate of one column every two hours (Wolff and Ready, 1991). This development can be visualized using monoclonal antibodies such as Mab22C10 (Zipursky et al., 1984) that specifically labels cells recruited as photoreceptor neurons (Figure 3B). Two domains of mitosis are seen in the developing third instar eye disc. The first mitotic event occurs ahead of the Morphogenetic furrow and it is asynchronous. The second mitotic wave is synchronized and occurs immediately behind the

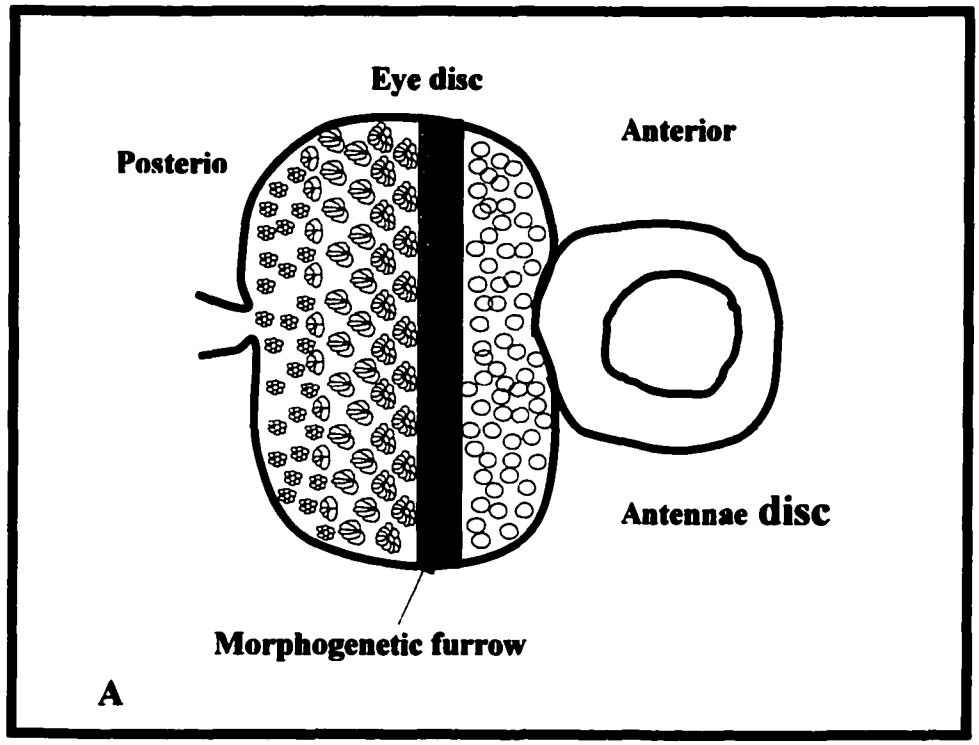


Figure 3. A) **Schematic diagram of the developing third larval instar eye disc.** Morphogenesis in the eye disc is marked by the formation of a groove known as the morphogenetic furrow (MF). The MF is a dynamic structure that moves from posterior to the anterior part of the eye disc. Cells ahead of the MF are undifferentiated, while cells behind the MF are recruited into a three dimensional array of photoreceptor and accessory cells. B) A third instar eye disc stained with the neuron specific antibody mAb22C10 reveals differentiating photoreceptor clusters posterior to the MF (white Arrow is pointing at the morphogenetic furrow).

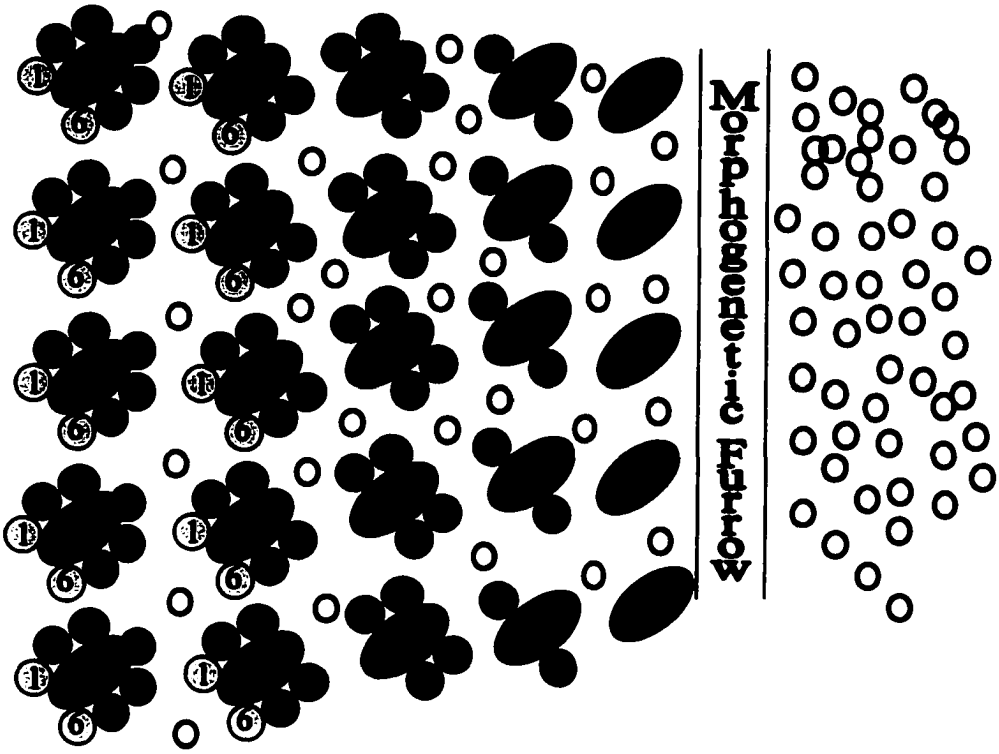


Figure 4. The photoreceptor cells develop in a stereotypical order. As the MF moves from posterior to anterior cells, behind the MF they are recruited into photoreceptor cells. The first cell to be recruited is photoreceptor cell R8 followed in a pair wise manner by R2, R5 and R3, R4. Subsequently R1 and R6 join the cluster followed by photoreceptor R7; the last cell to join the cluster.

pool of cells generated from the first mitotic event. Precursors for the R1, R6 and R7 photoreceptors are recruited from the pool of cells derived from second mitotic event. Several molecular markers have been used to study details of the differentiation of the photoreceptor cells (Zipursky et al., 1984). Such studies have concluded that the recruitment of the R-cells occurs in a stereotypical sequence. The first cell to differentiate is the photoreceptor R8, followed in a pair wise manner by R2, R5 and R3, R4. Subsequently, R1 and R6 differentiate, followed by photoreceptor R7, which is the last cell to join the cluster (Tomlinson and Ready, 1987). Once the photoreceptors cells are recruited, the cone cells join the clusters during the late third instar of larval. The remaining cells, pigment cells and cells of the mechanosensory bristle complex develop during the pupal stage (Cagan, 1993).

Signal Transduction Pathways and Pattern Formation in the Developing Eye.

Early experiments using genetic mosaics led to the idea that cell fate specification in the developing eye is directed by cell-cell interactions rather by strict lineage mechanisms (Lawrence and Green, 1979; Ready et al., 1976). Molecular genetic studies have led to the characterization of several important signal cascades that regulate cell fate determination and pattern formation in the developing eye. Initially, the sevenless (Sev) receptor tyrosine kinase (RTK) activated signaling pathway was characterized as a pathway specific for the determination of the R7 cells that gave rise to the idea that unique signaling pathways may be involved in directing specific cell fates. Subsequently, it was shown that the Sev signaling pathway could also induce non-R7 cell fates when expressed ectopically in the appropriate precursor cells (Zipursky and Rubin, 1994). Furthermore, another signal cascade mediated by EGFR (Epidermal growth factor

receptor) can direct the specification of an R7 cell fate in the absence of the Sevenless function. In the developing eye, activation of EGFR can direct the determination of all cell types (Freeman, 1996; Tio and Moses, 1997) and the EGFR signal cascade is involved in a number of cellular processes including MF initiation, cell proliferation, cell spacing and cell survival (Dominguez et al., 1998; Freeman, 1997; Freeman and Bienz, 2001; Lesokhin et al., 1999). The EGFR activated signaling pathway is involved in a myriad of developmental processes, including wing vein formation, midline-glial differentiation and establishment of axial polarity in the follicle cells (Clifford and Schupbach, 1992; Clifford and Schupbach, 1994; Freeman, 1994; Klambt et al., 1991; Neuman-Silberberg and Schupbach, 1993; Price et al., 1989; Raz and Shilo, 1992; Raz and Shilo, 1993; Roth et al., 1995; Schupbach and Roth, 1994; Sturtevant et al., 1993; Xu and Rubin, 1993). Both sevenless and EGFR transduce signals via a common evolutionary conserved downstream signal cascade, the RAS1-MAPK cascade, which includes RAS1, RAF and MAPK kinase (MEK) and MAPK (mitogen activated kinase). Signals transduced through this protein phosphorylation cascade lead to the activation of transcription factors which in turn regulate specific downstream targets in various cell types (Brunner et al., 1994; Dickson et al., 1992; Flores et al., 1998; Flores et al., 2000).

The signaling pathway activated by *Notch* (*N*), also plays many important roles during eye development. In the developing eye, *Notch* is involved in cell proliferation, cell fate determination, establishment of dorso-ventral axis formation and establishment of ommatidial polarity (Cagan and Ready, 1989; Cooper and Bray, 1999; Fortini and Artavanis-Tsakonas, 1994; Fortini et al., 1993; Papayannopoulos et al., 1998). N is activated by the transmembrane ligand, Delta (Dl). Activated N, combined with the

transcriptional regulator and Suppressor of Hairless [S (H)] activates downstream targets. In addition to its role in eye development, N signaling is critical for a large array of cell-cell interactions and cell fate decisions during *Drosophila* development reviewed by (Artavanis-Tsakonas et al., 1999).

Genetic studies in *Drosophila* have shown that cAMP and PKA (A kinase or Protein kinase A)-mediated signaling is required in a variety of processes, including oogenesis, establishment of tissue polarity in the embryo, imaginal disc morphogenesis and synaptic function (Davis et al., 1998; Davis et al., 1996; Lane and Kalderon, 1993; Lane and Kalderon, 1995). In the developing eye, PKA plays an important role in the initiation of pattern formation and morphogenesis through its interaction with Hedgehog (Hh), DPP and Wingless (Wg) (Burke and Basler, 1996; Dominguez and Hafen, 1997; Heberlein and Moses, 1995; Heberlein et al., 1995; Huang and Kunes, 1998; Pan and Rubin, 1995; Pignoni and Zipursky, 1997). Signaling by Sev, EGFR, N, and the cAMP-PKA pathways regulate a multitude of cellular functions in different tissues, and due to the number of transcription factors involved, it became apparent that a single signaling pathway alone could not specify particular cell fates. Recent studies have demonstrated that the determination of specific cell fates involves the integration of inputs from multiple pathways and a combinatorial code of activated transcription factors (Flores et al., 2000; Halfon et al., 2000; Simon, 2000; Tomlinson and Struhl, 2001; Xu et al., 2000).

Chapter II

Neuronal development in the *Drosophila* compound eye: photoreceptor cells R1, R6, and R7 fail to differentiate in the *retina aberrant in pattern* (*rap*) mutant.

Summary

The compound eye of *Drosophila* is a reiterated pattern of 800 unit eyes known as ommatidia. In each ommatidium there are eight photoreceptor neurons (R1-R8) and an invariant number of accessory cells organized in a precise manner. In the developing eye, specification of cell fates is triggered by sequential inductive events mediated by cell-cell interactions. The R8 photoreceptor neuron is the first cell to differentiate and is thought to play a central role in the recruitment of the remaining photoreceptor cells. Previous work from our laboratory demonstrated that mutations in the *retina aberrant in pattern* (*rap*) locus lead to abnormal pattern formation in the compound eye. Genetic mosaic experiments demonstrated that for normal retinal patterning to occur, *rap* gene function is required only in the photoreceptor cell R8 (Karpilow et al., 1989). In this study I have analyzed the R cell composition of developing as well as the adult eyes of *rap* mutants employing a variety of R cell specific markers. I show that in *rap* mutants, although some of the R8-specific markers show normal expression patterns, other aspects of the R8 cell differentiation are abnormal. In addition, the cells R1, R6, and R7 fail to differentiate properly in *rap* mutants. These results suggest that the *rap* gene encodes an R8-specific function that plays a role in the determination of the photoreceptor cells R1, R6, and R7 (Sigrist and Lehner, 1997).

Introduction

Our laboratory previously showed that mutations at the *retina aberrant in pattern (rap)* locus disrupt normal neuronal differentiation in the developing eye disc and consequently alter the number and organization of R cells within the adult ommatidia (Karpilow et al., 1989). In addition, a mosaic analysis showed that only ommatidia with a wild type (*rap*+) R8 cell were patterned in a normal configuration, consistent with the notion that the *rap* gene product encodes an R8-specific function important for normal ommatidial pattern formation (Karpilow et al., 1989). However, from our previous results it was not clear whether the aberrant patterning in *rap* mutants was an indirect consequence of the failure of R8 differentiation, or whether *rap* mutations affected a specific aspect of signaling function by a differentiated R8 cell.

In this chapter I have addressed these issues by analyzing the cellular composition of the retina in *rap* mutants. I employed a combination of genetic, molecular, and histochemical techniques to identify the different photoreceptor cell types present in the *rap* mutant. My findings show that in *rap* mutants, the R8 cell differentiates but is abnormal in certain aspects and is unable to carry out some of the signaling functions normally required for proper ommatidial assembly. As a consequence, the cells R1, R6, and R7 fail to differentiate and instead the precursors of these cells adopt a fate of cell death. These data suggest that *rap* gene function may be required for coordinating one or more R8-specific functions and provide evidence for the requirement of R8 in the differentiation of R1 and R6 cells in addition to its well-known role in R7 cell determination (Sigrist and Lehner, 1997).

Results

Strategy for Identifying R8 in Adult *rap* Retina.

In the wild type *Drosophila* compound eye, the different R cells can be identified based on their positions within the ommatidia and their rhabdomere size [Figure 5 (A, C), 2(A, B)]. The disorganized arrangement of the adult *rap* retina precludes the identification of photoreceptor cell types using these criteria [Figure 5 (B, D), 2(E, F)]. To circumvent these problems and to determine whether R8 was present in the adult retina of *rap* flies, the *rap3* allele (a putative null allele) was crossed into a genetic background that either marked or eliminated photoreceptor cells R1-R7. To accomplish this, two mutations, *outer rhabdomeres absent* (*ora*) and *sevenless* (*sev*) were used. *ora* serves as an effective marker of photoreceptor cells R1-R6 and has been used extensively in the analysis of R cell types in combination with other mutations (O'Tousa et al., 1989). Following eclosion, the R1-R6 rhabdomeres in *ora* mutants slowly degenerate and are essentially absent after 14 days. The *ora* mutation does not perturb the organization and structure of the rhabdomeres of the R7 and R8 cells and thus allows the identification of these two cell types in the *rap* retina [Figure 6 (C, D)]. To distinguish R8 from the UV -sensitive photoreceptor R7, the *sev* mutation was crossed into to the *rap; ora* genetic background. The *sev* mutation eliminates the R7 cell by directing the R7 precursor cell to a cone cell fate. The *sev* mutation exhibits no known effect on the remaining R cells (Harris et al., 1976; Tomlinson and Ready, 1986). Thus in a *sev; ora* double mutant retina, only R8 cells will exhibit intact rhabdomeres. The presence or absence of R8 cells in *rap* mutants was thus determined by serially sectioning the retina of adult *rap. sev; ora* triple mutant flies and scoring ommatidia for

the presence of intact rhabdomeres. Similarly, the number of R7 cells can be estimated by comparing the structure of ommatidia in *rap, ora* double mutant with those of the *rap, sev; ora* triple mutant. These double and triple mutants were constructed by standard genetic crosses and were aged 16- 20 days. Subsequently, the eyes of these flies were serially sectioned and scored at each level for the presence of normal R cells with intact rhabdomeres.

Analysis of *rap;, sev; ora:* Triple Mutant Shows That *rap* Retina Contains R8 Cells

Using light microscopy, the phenotype of 179 ommatidia from eight *rap, sev; ora* triple mutant eyes were scored for the presence of R cells with intact rhabdomeres; of these 160 out of 179 ommatidia (89%) contained at least one R cell with an intact rhabdomere. Thus, while cell number and photoreceptor positioning are disrupted in *rap* mutants, these abnormalities do not perturb the ability of R8 cells to differentiate [Figure 6 (G, H)]. It is worth noting that the position of the putative R8 rhabdomere varied greatly from one ommatidium to the next. Unlike the morphology observed in the wild type eye, where R8 is centrally located within the boundaries of each ommatidium and is confined to the basalmost half of the retina, the rhabdomeres of the triple mutant were often situated peripherally (in positions where the rhabdomeres of R1-R6 normally reside) and were distributed in both the apical and basal regions of the retina. As a consequence of the variable positioning of the R8 rhabdomere, no single section was ever observed in which all of the ommatidia contained an intact rhabdomere. This necessitated a 3-dimensional analysis of the triple mutants using adjacent serial sections.

Figure 5: Morphology and anatomy of the *rap* mutant eyes compared with wild type eyes. (A) Scanning electron micrograph of the wild type compound eye showing a smooth array of hexagonal unit eyes, the ommatidia (orig. mag. X 185). (B) *rap* mutants exhibit a rough exterior surface due to the uneven size and shape of the ommatidia (orig. mag. X210). (C) Tangential sections of the wild type eye shows the invariant number and organization of the photoreceptor cells (R Cells) in each ommatidium. *(D) rap mutants* show variable number and position of the R cells. Scale bar = (C, D) 50 μm

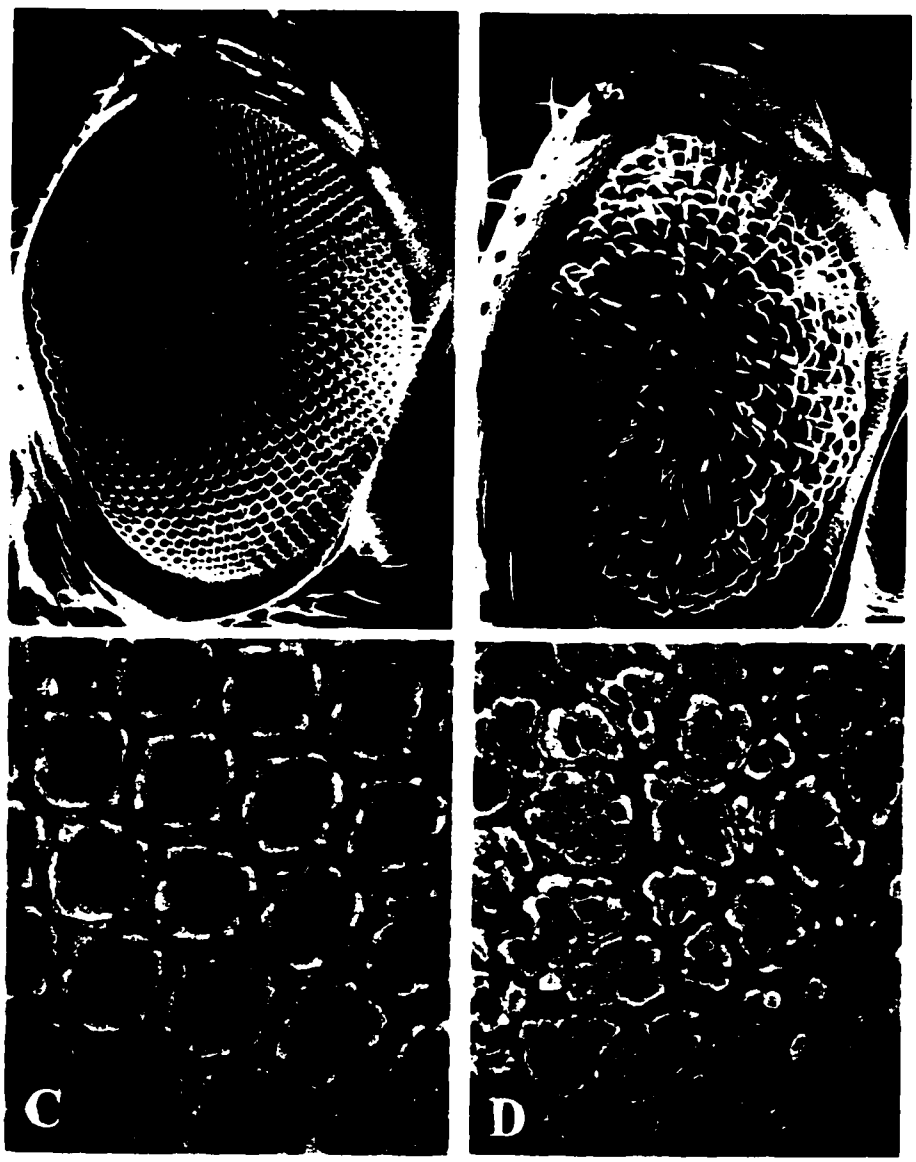


Figure 6. In the wild type eye the photoreceptor cells can be identified by their invariant position within the ommatidia (A) A tangential section of the wild type eye viewed under a light microscope. (B) The organization of a single ommatidium as seen through the transmission electron microscope. The different R cells can be identified by their stereotypic position within an ommatidia. (C) Tangential sections through an *ora* mutant eye at the level of the R7 cell. (D) Transmission electron micrograph of single *ora* mutant ommatidium shows an intact R7 cell with its rhabdomere and degeneration of the rhabdomeres in cells RI-R6. (E) Light and (F) transmission electron micrographs of *rap* ommatidia showing abnormal position and number of R cells. This prevents identification of the different R cell types. (G, H) Anatomy of the *rap, sev; ora,* triple mutant ommatidia. (H) A tangential section of a triple mutant eye seen through light microscope and (G) transmission electron micrograph of a triple mutant ommatidium showing a single central R8 cell with an intact rhabdomere. Scale bar = (A, C, E, G) 50 μm; (B, D, F, H) original magnification x9000.

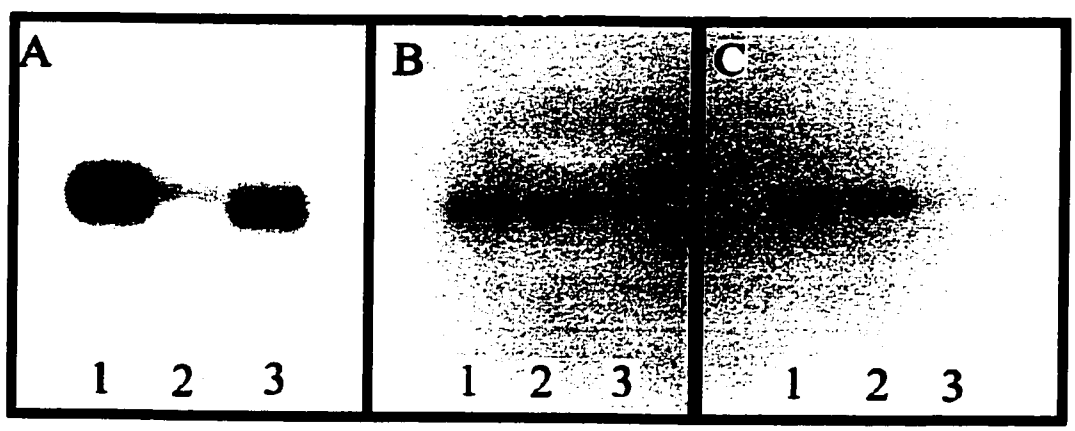
rap; ora Double Mutants Show that _rap_ Retina Lacks Majority of R7 Cells.

I next wished to determine whether *rap* mutants contained a normal complement of the R7 cells. To do this, I constructed *rap; ora* double mutants and estimated the number of central small rhabdomere R cells (R7- and R8-like) in serial sections. My expectation was that if the *rap* mutation did not affect R7 differentiation, then each ommatidium would have two central cells, an R7 and an R8 cell. The data from the double mutants were compared with the data from the *rap, sev; ora* triple mutants to estimate the number of R7-like cells in *rap* mutant eyes. Of the 111 *rap; ora* ommatidia examined, 101 (90%) contained only one cell with a small intact rhabdomere. Surprisingly, only a small percentage (10%) of *rap; ora* double mutant ommatidia contained two central cells with intact rhabdomeres. This is not substantially different from the data generated from the *rap, sev; ora* triple mutant (11%). These results suggest that R7 is not present in a majority of *rap* ommatidia.

Analysis of Opsin Transcripts in _rap_ Mutants.

Anatomical analysis of the double and triple mutants presented above suggested that *rap* flies contained only a small fraction of the normal R7 population. To investigate this further, the levels of various R-cell specific opsin transcripts were examined in *rap* mutant eyes. Three distinctive opsins, have been reported to be expressed in the compound eye of *Drosophila.* The first, Rh1, was previously shown to be specific to the R1-R6 class of photoreceptors (O'Tousa et al., 1985; Zuker et al., 1985). In contrast, Rh3 and Rh4 opsins were shown to be confined to mutually exclusive subsets of the

Figure 7. *rap* mutants show reduced levels of opsin mRNA. Northern blot analysis of opsin transcripts. The RNA blot was probed with (A) Rh I, (B) Rh3, and (C) Rh4 opsin probes. Lane 1: wild type (Oregon-R); lane 2: ora^{JK84}; lane 3: rap^{3}. The same blot was probed with actin as an internal control to ensure that an equivalent for the amount of RNA was loaded in each lane on the filter (not shown).



R7 photoreceptors (Montell et al., 1987; Zuker et al., 1987). A small but significant level (10- 15%) of the Rh3 transcript is also expressed in cells outside the retina. Subsequent to this work the R8 opsin Rh4 and Rh5 have been described (Papatsenko et al., 1997; Salcedo et al., 1999). To look for R1-R6 and R7 specific opsin transcripts in *rap* mutants, poly(A)$^+$ mRNA was isolated from adult *rap* fly heads analyzed on Northern blots and compared to wild type (Oregon-R) and *ora* mutants. The *ora* mutants contain two mutations (O'Tousa et al., 1989). One of these mutations is in the *ninaE* locus, which is the structural gene for the Rh1 opsin. *ora* mutants have been shown to express Rh1 at about 10% of the wild type levels (O'Tousa et al., 1985). Northern blots were quantified on the AMBIS Radioanalytic System and compared with levels of actin transcript to accurately quantify opsin mRNA levels. Figure 7 (A) shows the data from my analyses of Rh1 opsin levels in wild type (*Oregon-R*), *ora* and *rap* flies. When compared to wild type flies, the *rap* flies contained a significantly lower level (60%) of the R1-R6 specific transcript (Rh1). This finding suggests that *rap* mutants lack a significant fraction of the R1-R6 class of photoreceptors. This is supported by my analyses of the cell types in the developing *rap* discs (see below). *ora* mutants served as negative controls and, as expected, showed only 10% of normal Rh1 levels. When blots probed were with R7-specific opsins [Figure 7 (B, C)], wild type and *ora* flies showed similar amounts of the Rh3 and Rh4 transcripts. In contrast, *rap* flies contained only 18 and 14% of the wild type R7-specific opsin mRNA levels, respectively [Figure 7 (B, C)]. These results suggest that *rap* mutant eyes lack a majority of the R7 cells and support data from the anatomical studies on the double and triple mutants described above.

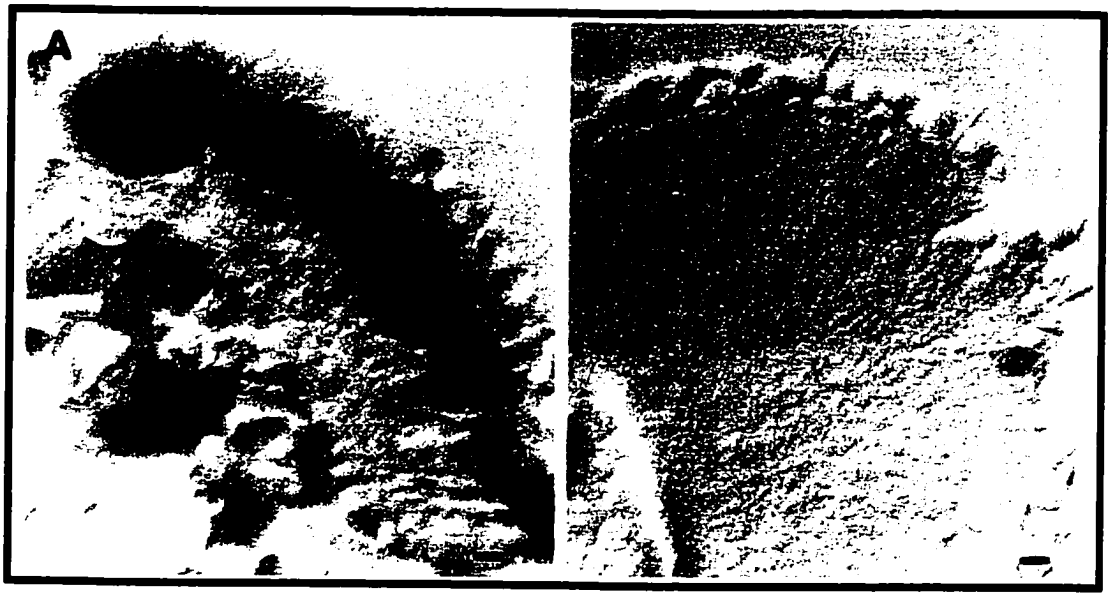
Rh4-lacZ Expression in *rap* Mutants

To further substantiate the Northern analysis data presented above, I used a *Rh4-lacZ* transgenic line (Fortini and Rubin, 1990) to detect the Rh4 expressing R7 cells in *rap* mutants by assaying β-Gal expression. The rap^{X3} mutation (a null allele) was crossed into the *Rh4-lacZ* background and frozen sections of the heads from *rap / rap; Rh4-lacZ* and *rap / +; Rh4-lacZ* flies assayed for β-Gal expression. In the *rap* mutant background no Rh4 - driven β-Gal expression was seen [Figure 8 B]: In flies heterozygous for the *rap* mutation, wild type levels of the Rh4 driven β-Gal expression is seen [Figure 8 A]. These data taken together confirm that *rap* mutant eyes lack a majority of the R7 cells and about 30-40% of the R l-R6 subclass of photoreceptors.

Studies of R-Cell Types in Developing Eye Imaginal Disc of *rap* Mutants

To determine whether the lack of the specific sub-sets of R cells in the *rap* adult eye was due to failure or improper differentiation of these cells during eye development, I examined the R-cell composition of developing third instar eye imaginal discs. Previous studies with the neural-specific monoclonal antibody mAb22C1O showed that the R-cell patterning was abnormal in *rap* mutants (Karpilow et al., 1989). In this study, I employed a variety of R-cell specific markers to specifically label the differentiating cells in the developing eye disc.

Figure 8. ***rap*** **mutants lack majority of the Rh4 expressing R7 cells.** Cryostat sections of fly heads showing Rh4 opsin promoter driven β-Gal expression in (A) *rap/+; Rh4-lacZ* strain and (B) *rap / rap; Rh4-lacZ* mutants. Scale bar = 50μm



Cobalt Sulfide Staining.

In the developing eye disc the earliest morphogenetic events immediately posterior to the morphogenetic furrow can be visualized by staining the eye imaginal disc with cobalt sulfide which highlights the cell membranes of the R-cells. In wild type discs [Fig. 9 (A)], rosettes of cells pinch off from the furrow and a cell from the core of this rosette becomes an R8 cell as seen by the darkening of the cell outlines (Wolff and Ready, 1991). In more posterior regions, three cell clusters comprising R8, R2, and R5 can be seen, followed by the recruitment and differentiation of R3 and R4 that results in five cell clusters. In *rap* mutant discs, cobalt sulfide staining reveals abnormally sized clusters immediately posterior to the furrow. Clusters with one and frequently more than one R8 cell are seen (Figure 9). However, the precise spacing of the differentiating R-cell clusters does not seem to be affected in the *rap* mutant. These data suggest that the early steps in the R-cell determination pathway are perturbed in *rap* mutants leading to abnormal patterning and differentiation of the R8 cell. The criteria for identifying the specific R cells in cobalt stained preparations is based on the spatial position of the differentiating cell within the clusters and the precise position of the cluster in relation to the morphogenetic furrow.

Scabrous Expression Reveals Early Stages of R8 Specification in rap Mutants.

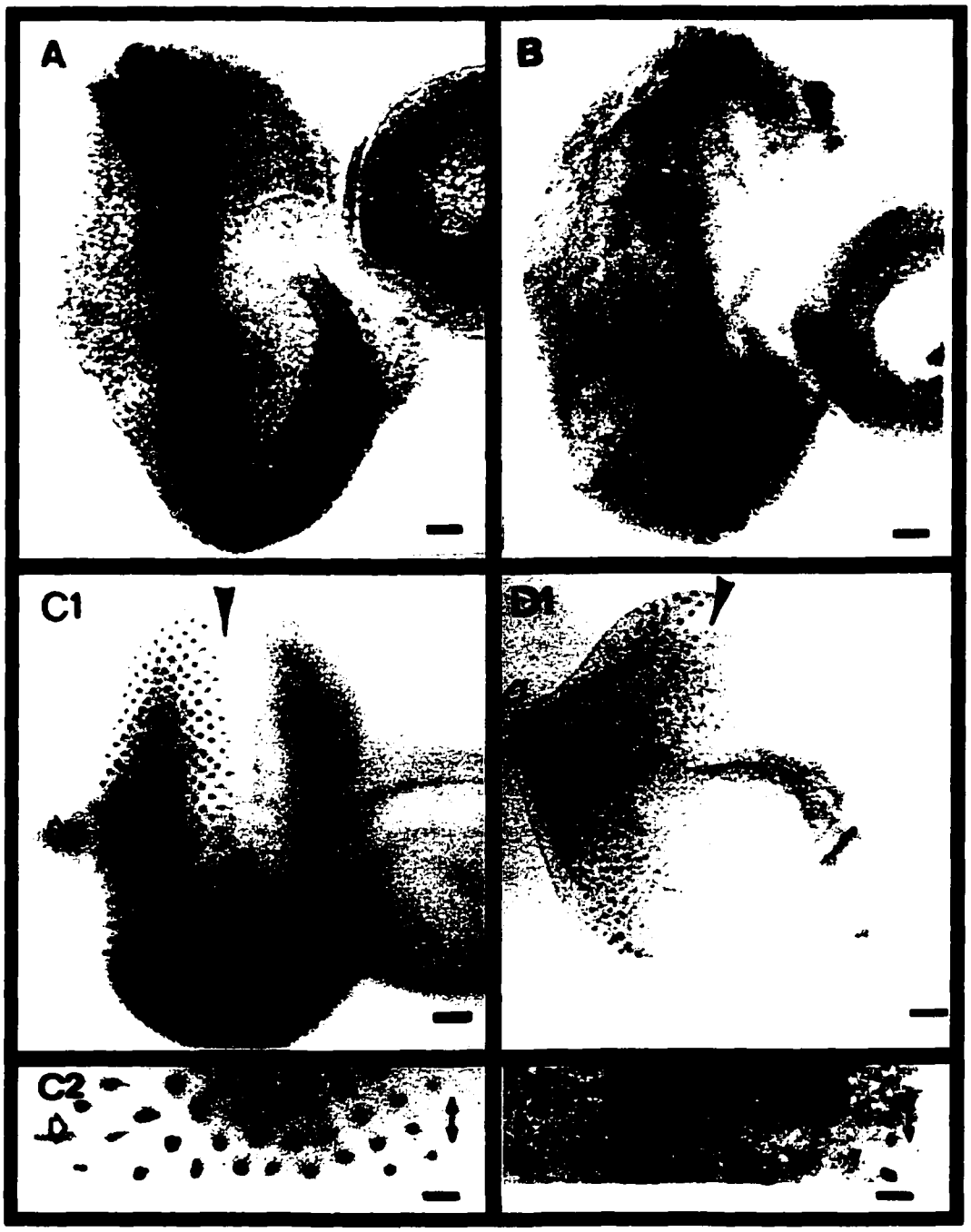
The *scabrous (sca)* gene encodes a fibrinogen like secreted polypeptide that is thought to mediate cellular spacing during differentiation of the R8 cells (Baker et al., 1990; Baker and Zitron, 1995; Mlodzik et al., 1990a). In third instar eye discs, Sca expression is initially restricted to R8 cells immediately behind the furrow (Baker et al., 1990). I used anti-Sca antibody to visualize R8 cells in *rap* mutant discs. In wild

Figure 9. Cobalt sulfide staining reveals early pattern defects in *rap* mutants. Late third instar stage eye discs from (A) wild type (Oregon-R) and (B) *rap* mutants were stained with cobalt sulfide as described in the Materials and Methods. The arrow marks the position of the morphogenetic furrow. (B) Arrowheads show the aberrantly sized clusters of presumptive R cells in *rap* mutants. Anterior to the right. Scale bar = 25 μm.

type eye discs, Sca protein is localized in R8 cells in about two to three rows of ommatidial clusters posterior to the morphogenetic furrow [Figure 10 A]. In *rap* discs, Sca protein is expressed in a pattern similar to the wild type spacing and differentiation of the R8 cells [Figure 10 (B)]. This is consistent with the results from the cobalt staining experiments that showed that the spacing of the presumptive R-cell clusters behind the morphogenetic furrow was not affected by the *rap* mutation.

Boss Protein Expression Is Abnormal in rap Mutants. The Boss protein is expressed specifically in R8 cells and is the inductive signal for R7 determination (Kramer et al., 1991; Reinke and Zipursky, 1988). Kramer *et. al.* demonstrated that the expression of the Boss protein in R8 is initiated shortly after the passage of the morphogenetic furrow and in more posterior regions of the disc, Boss protein is internalized into the R7 precursor cell. The internalized Boss protein can be visualized using anti-Boss antibodies. To determine whether the lack of R7 cells in *rap* mutants was due to abnormalities in the R8 cell, I used anti-Boss antibodies to identify R8 cells in the developing wild type and *rap* discs. In wild type discs, Boss expression is seen in R8 cells behind the morphogenetic furrow and in more posterior regions of the disc, and internalized Boss protein can be seen in the presumptive R7 cells [Figure 10 C]. In *rap* eye imaginal discs, however, anti-Boss antibody staining is abnormal. Initially Boss protein is seen in R8 cells at a short distance behind the morphogenetic furrow in rows of single cells [Figure 10 D1]. This pattern is maintained for approximately five to seven rows, and then the staining appears to be greatly reduced or absent in the more posterior regions of the imaginal disc morphogenetic furrow [Figure 10 (D2)]. In *rap* discs Sca protein is

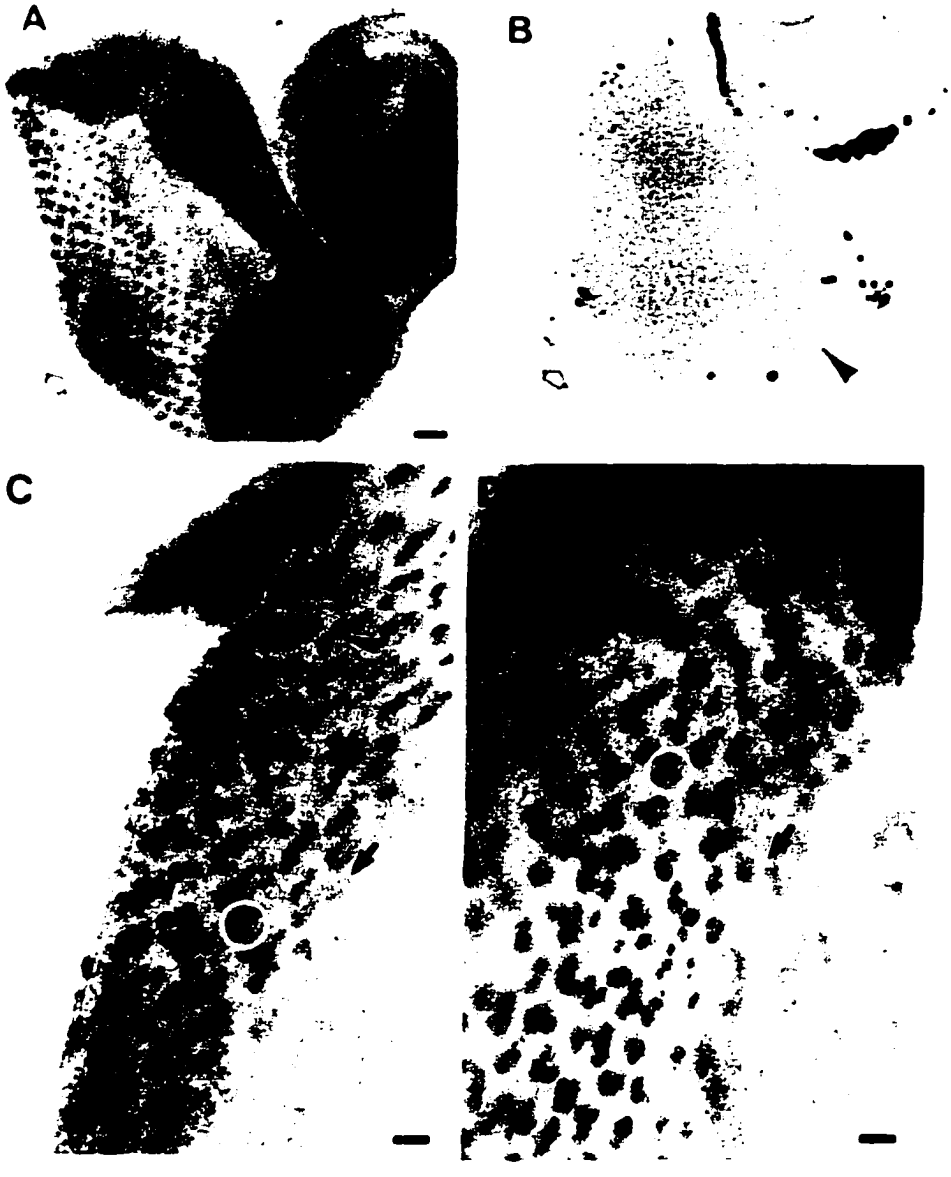
Figure 10: R8 differentiation is revealed by various molecular markers in the third instar eye disc. R8-specific *scabrous* expression is similar in (A) wild type and (B) *rap* mutant third instar eye discs. Boss protein expression in the developing eye disc. In the wild type disc Boss is localized to R8 cells starting a few rows posterior to the morphogenetic furrow (arrowhead) and extends until the posterior end of the disc. (C2) At higher magnification Boss can be seen internalized in the presumptive R7 cell (arrowhead). In *rap* mutant discs Boss distribution is abnormal. (D1) Boss expression initiates normally but becomes aberrant in the posterior regions of the disc. (D2) Higher magnification views of the posterior regions of the disc shows Boss expression to be reduced or absent. Anterior to the right. Open arrowheads point to the optic stalk region of the disc. Scale bar = 50 μm.



expressed in a pattern similar to the wild type spacing and differentiation of the R8 cells [Figure 10 B]. This is consistent with the results from the cobalt staining experiments that showed that the spacing of the presumptive R-cell clusters behind the morphogenetic furrow was not affected by the *rap* mutation.

Abnormal R8 Cell Differentiation Revealed by Labeling with Enhancer Trap AC65N. I used an enhancer trap line AC65N, which expresses β-Galactosidase only R8 cells, to follow R8-cell differentiation in *rap* eye discs (L. Zipursky and U. Banerjee, personal communication). In the wild type background, AC65N is expressed in R8 cell thus revealing an expression pattern that begins three to four rows posterior to the morphogenetic furrow and continues to be expressed in the posterior most regions of the eye disc [Figure 11A]. In the antennal disc, several groups of cells express β-Gal including those that map to regions of the zahnborsten, the arista, and the first antennal segment. As shown in figure 11 (B), the β-Gal expression pattern of *rap;* AC65N homozygotes differs from that of the parental AC65N line. The expression pattern in the antennal disc is unaffected in *rap;* AC65N discs and thus serves as an internal control. In contrast, only a small fraction of the R8-specific stain was observed in the eye imaginal disc. Thus *rap* mutants fail to express the AC65N β-Galactosidase in a tissue-specific manner. This suggests that although the R8 cell type is specified correctly, some aspect of R8 differentiation is perturbed in *rap* mutants.

Figure 11. (A) The enhancer trap line AC65N expression is R8 specific in the eye disc and stains distinct groups of cells in the antennal disc. (B) In *rap* mutants the R8-specific expression of the AC65N line in the eye disc abolished but its antenna specific expression is maintained. Arrowheads mark the morphogenetic furrow. Scale bar = 50 *μm*. R2, R5, and R8 cell differentiation visualized by *rhomboid (rho)* expression. (C) In wild type eye discs *rho* is specific to R8, R2, and R5. Its expression is strong in the R8 cell (arrowheads) and flanked by the weaker staining in R2 and R5 cells, resulting in a pattern of three cell clusters (circles). (D) In *rap* mutant eye disc *rho* expression labels R2, R5, and R8. The spatial arrangement of the cells is frequently altered and often reveals R8 cells adjacent to each other (arrowheads) .The arrow marks the position of the morphogenetic furrow. Scale bar = 25 *μm*



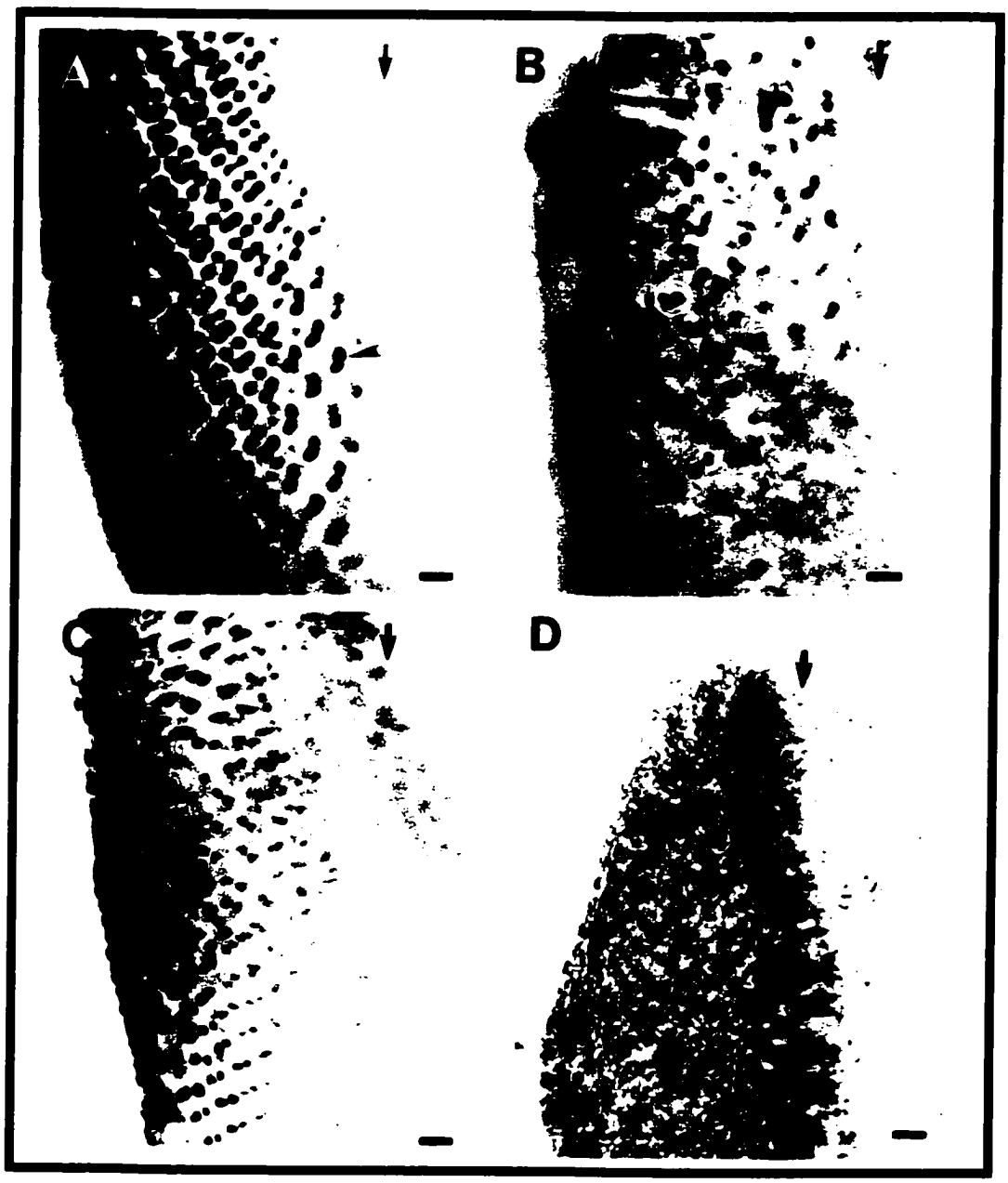
Differentiation of R8, R2, and R5 in rap Mutants Is Revealed by rhomboid Expression.

The *rhomboid* gene belongs to the *spitz* group of genes and is thought to act through the signaling pathway mediated by the epidermal growth factor receptor (Sturtevant et al., 1993). The enhancer trap line *rhoX81* expresses β-Galactosidase in cells R8, R2, and R5. I used the rho-81 enhancer trap line to label cells R8, R2, and R5 in the developing eye discs of *rap* mutants. In the wild type background, β-Galactosidase expression is seen in R8, and less strongly R2 and R5 [Figure 11C] .The expression pattern in the *rap* mutant background reveals the presence of the cells R2, R5, and R8. However, in *rap* mutants the array of R-cell clusters is highly disorganized, in some clusters an R8 cell is lacking, and in other clusters there is more than one R8 cell [Figure 11D]. These results suggest that while cells R8, R2, and R5 are formed in *rap* discs, their precise positioning and arrangement is disturbed.

R1 and R6 Differentiation Is Aberrant but Not R3 and R4 in rap Mutants.

The *seven up* gene product is a transcription factor belonging to the steroid receptor family and is required for the normal differentiation of cells R3, R4, R1, and R6. *Sevenup* expression begins initially in R3 and R4 cells posterior to the morphogenetic furrow and, subsequently, expression can be seen in cells R1 and R6 that results in a characteristic labeling of a four cell pattern (Hiromi et al., 1993; Mlodzik et al., 1990b). To test if the differentiation of R3, R4, R1, and R6 cells was normal in *rap* mutants, I used a *seven up* enhancer trap line to specifically label these cells in a *rap* mutant background. In the wild type background, β-Gal expression can be seen initially in R3 and R4, followed by the appearance of R1 and R6 [Figure 12 A].

Figure 12. R3, R4, R1, and R6 differentiation studied by *sevenup (svp)* and Bar H1 expression. (A) The sv*p* is initially expressed in the cells R3 and R4 (arrowhead) and subsequently R1 and R6 express *svp* giving rise to a four cell pattern (circle). (B) In *rap* mutant discs sv*p* can only be seen in R3 and R4 (circle) .R1 and R6 fail to differentiate (C). In the wild type disc R1 and R6 cells can be detected by the specific Bar HI expression, whereas (D) in *rap* mutants there is no Bar HI expression due to the lack of R1 and R6 cells. The arrow marks the position of the morphogenetic furrow. Anterior to the right. Scale bar = 25 μm.



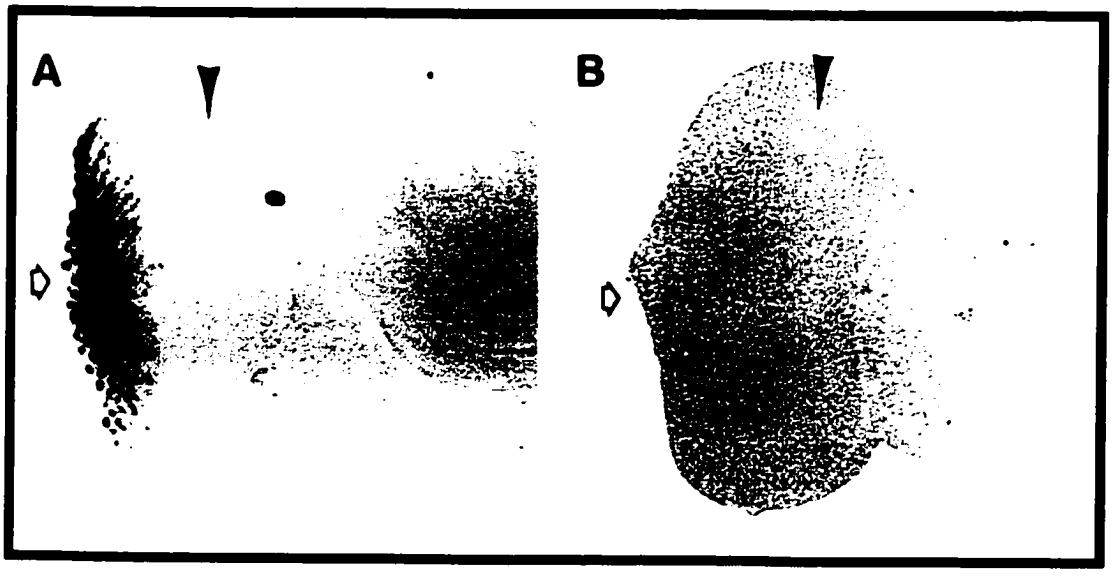
In contrast, in a *rap* mutant background β-Gal expression can be initially seen in R3 and R4, but subsequently R1 and R6 are not seen [Figure 12 B]. These results suggest that in *rap* mutants, although R3 and R4 differentiate properly, R1 and R6 cells fail to be recruited into the pattern of the developing eye.

Expression of Bar H1 Protein Shows That R1 and R6 Fail to Differentiate in rap Mutants.

To further investigate the fate of R1 and R6 cells in the developing *rap* eye discs, I employed anti-Bar antibody as a cell-specific marker to label the R1 and R6 cells. In wild type eye imaginal discs the bar protein is initially expressed in a group of undefined cells near the morphogenetic furrow. Subsequently, Bar expression is specifically localized in R1 and R6 cells at the posterior edge of the wild type disc, resulting in a chevron-like staining pattern [Figure 12 C]. In third instar *rap* mutant eye discs, anti-Bar antibodies fail to localize the R1 and R6 cells, suggesting that these cells failed to fully differentiate [Figure 12 D].

Abnormal Differentiation of R7 Cells in rap Mutants. To localize R7 cells in the developing eye disc I used the XA12 enhancer trap line as a cell-specific marker. In the XA12 enhancer trap line, β-Gal expression can be seen in the presumptive R7 cells in the posterior regions of the third instar eye disc [Figure 13 A]. In a *rap* mutant background, the R7 specific expression of β-Gal is absent [Figure 13 B]. These observations suggest that R7 differentiation is abnormal in *rap* mutants and supports my earlier data from anatomical studies with the double mutants as well as the opsin analyses presented above.

Figure 13. R7 cells fail to differentiate in *rap* mutants. (A) The enhancer trap line XA12 stains R7 cells in the third instar eye disc. (B) In a *rap* mutant background XA12 fails to detect R7 cells. Arrowheads mark the position of the morphogenetic furrow. Anterior to the right. Scale bar = 50μm.



rap Mutants Show Increased Cell Death. During the development of the *Drosophila* eye, excess precursor cells are eliminated at larval as well as pupal stages by programmed cell death (Wolff and Ready, 1991). Acridine orange staining is a simple and reliable method to detect cells undergoing programmed cell death (Bonini et al., 1993; Wolff and Ready, 1991). To ask whether the differentiation failure of R1, R6, and R7 in *rap* mutants results in a change in the pattern of cell death, I stained third instar eye discs with acridine orange. In wild type discs [Figure 14 A] there is usually variable and low-level cell death near the morphogenetic furrow (Wolff and Ready, 1991). In *rap* mutants there is a marked increase in cell death posterior to the morphogenetic furrow [Figure 14 B]. This increase in cell death seen in *rap* mutants may be due to the elimination of the precursor cells that were destined to become R 1, R6, and R 7.

***rap* mutants show abnormal number of cone cells as well as pigment cell.**

To determine if defective patterning leads to abnormal number of cone and pigment cells, *rap* mutant 48 hours pupal eye imaginal disc were dissected and stained with the antibody against the armadillo (arm) protein. The armadillo protein is found in the adhesive junction where it joins epithelial cells and other cells (Peifer and Wieschaus, 1990). This protein is expressed in the pigment cells, cone cells, and photoreceptors where they contact each other (Ahmed et al., 1998; Peifer and Wieschaus, 1990). In the wild type eye disc, anti-Arm stained the four cone cells and the 6 pigment cells of each ommatidia (Figure 15). However, the *rap* mutant allele rap^{X3} shows that there is variable number of cone cell as well as pigment cells.

Figure 14. Cell death in (A) wild type and (B) *rap* mutants visualized by Acridine orange staining. Wild type discs show a small amount of variable cell death near the furrow. In *rap* mutant discs a marked increase in cell death is seen posterior to the furrow. Scale bar = 50 μm.

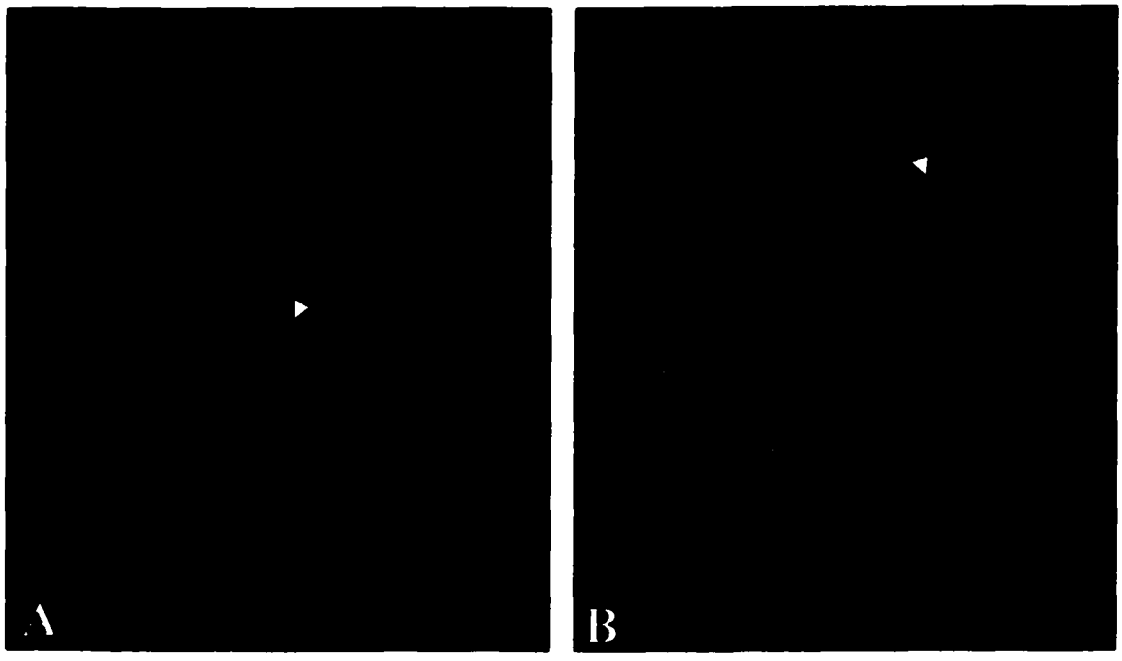
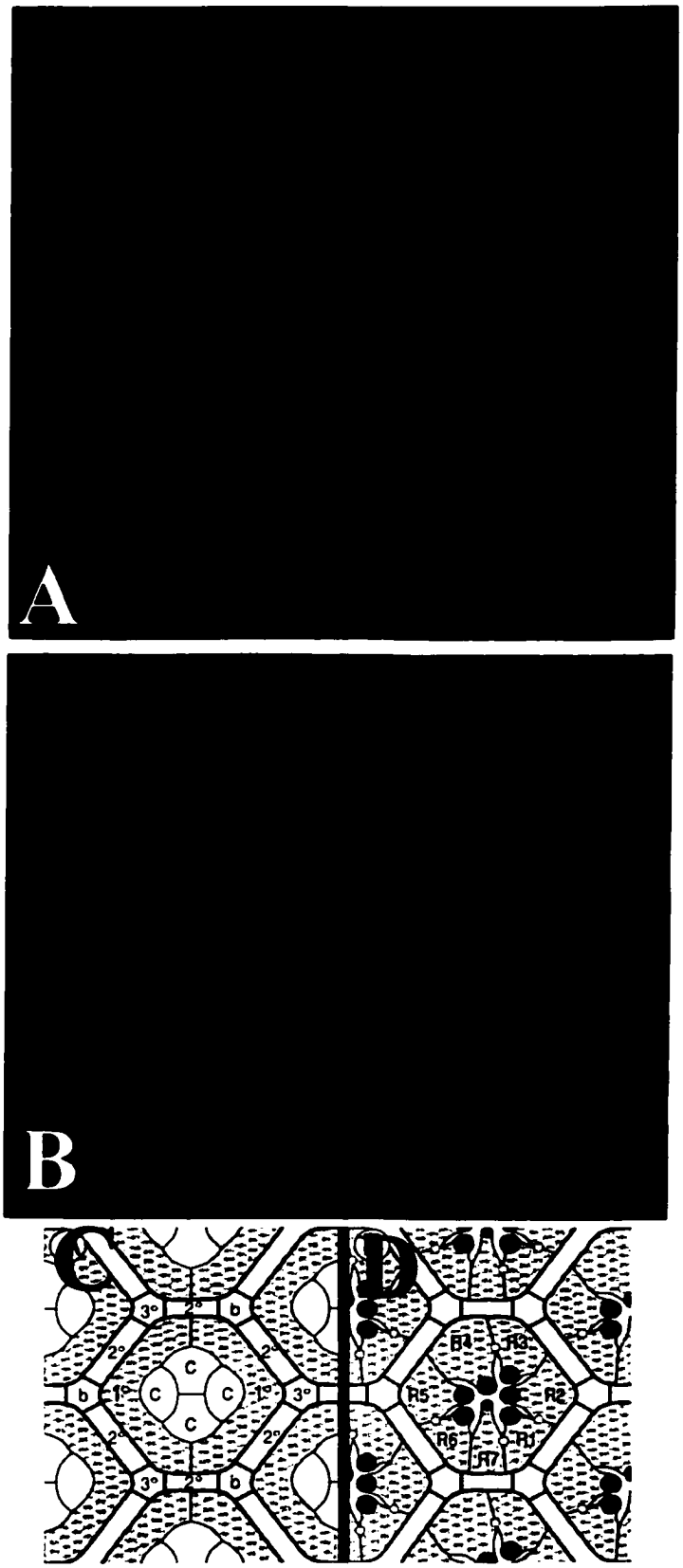


Figure 15. *rap* mutants show defects in the number of cone cells as well as pigment cells. *CS* (A) and rap^{X3} (B) eye discs were stained with anti-armadillo antibody. A diagram of the normal pattern of the cone cells and pigment cells are shown in C. In the wild type, each ommatidium contains 4 cone cells and 6 pigment cells (A). In rap^{X3} allele, a variable number of cone cells (arrows in B) as well as a variable number of pigment cells are seen (asterisks in B).



Discussion

During the development of the *Drosophila* eye, cellular determination and pattern formation takes place by sequential induction of cell fates. In this process the R8 cell differentiates first, and consequently the R8 cell is thought to play a central role in the induction of the remaining R cells through precise cell contacts. The inductive role of R8 in the specification of the R7 cell has been clearly demonstrated (Zipursky and Rubin, 1994), and several genetic functions required in the R8 cell have been characterized (Baker et al., 1990; Jarman et al., 1994). However, the precise role of the R8 cell in the determination of the remaining R cells is not yet clear. Our previous studies showed that *rap* mutations result in a rough eye phenotype due to the variable number of R cells in the ommatidia. In addition, a mosaic analysis showed that for normal pattern formation, only the R8 cell was required to be rap^{+}, suggesting an R8-specific function that is important for the proper differentiation of R cells (Karpilow et al., 1989). In this thesis I have addressed the question of whether the pattern abnormalities observed in the adult *rap* retina are a consequence of the lack of photoreceptor R8 or alternatively whether R8 differentiates but yet is defective in some function that is necessary for normal ommatidial organization. To address this issue I used several methods to identify the various R-cell types in the adult as well as in developing eyes. My results show that in *rap, sev; ora* triple mutants where only R8 cells should have an intact rhabdomere, a single R cell with an intact rhabdomere can be identified in a majority of the ommatidia. Assuming these three mutations act in an additive fashion, I infer from

these results that a mutation in the *rap* gene does not alter at least some of the steps in differentiation of the R8 precursor into an adult photoreceptor. Furthermore, anatomical studies of the double mutants *(rap; ora)* and analyses of the opsin transcripts in wild type and mutant flies suggest that the *rap* mutants lack a substantial fraction of the R1- R6 subgroup as well as a majority of the R7 cells. To investigate the developmental basis of this phenotype, I used molecular markers to identify the different R-cell types in the third instar eye discs. My experiments were based on the rationale that either the lack of, or the aberrant expression pattern of an R-cell specific marker is suggestive of defects in the differentiation of the particular R-cell type. More complicated interpretations, however, can be made. My results show that in *rap* discs the R8 cell differentiates but appears to be abnormal, as judged by the cobalt sulfide staining and aberrant expression patterns of Boss, *rhomboid,* and the R8-specific enhancer trap line AC65N. In addition, R2, R3, R4, and R5 cells appear to be specified in a normal manner. In contrast, R1, R6, and R7 cells fail to differentiate, supporting my data from the studies on adult eyes. In the developing eye disc, the precursor cells for R1, R6, and R7 are generated by the second wave of mitosis; it is unclear whether the failure of these cells to differentiate in *rap* mutants is due to defects in the mitotic cycle. My preliminary observations show that there is a marked increase in cell death in *rap* mutants as evidenced by acridine orange staining of the eye discs, further supporting the notion that surplus undifferentiated precursor cells may be directed toward a regulated fate of cell death. However, the nature of the identity of the dying cells is not clear from my data. Programmed cell death (apoptosis) has been shown to regulate the cell number in the

developing *Drosophila* eye by eliminating the excess undetermined cells (Wolff and Ready, 1991).

My results taken together suggest that the *rap* gene encodes an R8-specific function that plays a role in the differentiation of R1, R6, and R7. Although the role of R8 in R7 determination is well established, this is the first report demonstrating the role of R8 in the differentiation of R1 and R6. The *rap* gene function may be directly involved in the induction and/or survival of the cells R1, R6, and R7. On the other hand, a *rap* gene product may be required in the maintenance of the R8 differentiated state, for instance, by regulating the expression of some of the R8-specific genes like *boss* and *AC65N*. The abnormal positioning of the R8 cell in *rap* mutants as seen by cobalt sulfide staining may result in aberrant cell-cell contacts within the ommatidial clusters, precluding the subsequent inductive events necessary for subsequent recruitment of R1, R6, and R7 cells. In this model, the failure of R1, R6, and R7 differentiation in *rap* mutants may be indirect due to defects in the R8 cell itself. Such indirect effects due to perturbation of R8 development have been shown to disrupt R7 development.

Materials And Methods

Fly Stocks

Balancer chromosomes *In (3LR) TM813(4)^{DTS} th st* S *e* and *In(3LR) TM6 Mo Sb sr h D^{3} e* used to construct double and triple mutants were obtained from Dr. John Postlethewait at the University of Oregon. *ora^{JK84}* was obtained from J. O'Tousa at the University of Notre Dame and *v .sev^{LY3} m* was sent to us from the Benzer laboratory at the California Institute of Technology, Pasadena. *rap^{X2}* and *rap^{X3}* are X-ray induced alleles that behave as null alleles and were isolated in a mutagenesis screen by their failure to complement the hypomorphic allele *rap^{1}* (Karpilow et al., 1989). Double and triple mutants stocks were constructed by standard genetic crosses using the *rap^{3}* allele, which behaves as a null allele by genetic criteria. Homozygous enhancer trap lines AC65N *P(ry^{+}lacZ)ry^{-}* (3L-68C/D)and XAI2 *P(ry^{+}-lacZ)ry^{-}* (3R-94C / D) were generously donated by L. Zipursky (University of California, Los Angeles). The *sevenup* enhancer trap line, *svp* H 162 was obtained from Y. Hiromi at Princeton University. An enhancer trap line rhoX81 for the *rhomboid* locus was obtained from Ethan Bier (University of California, San Diego).

Antibodies and DNA Probes

Anti-Scabrous antibodies were obtained from Nick Baker (Albert Einstein College of Medicine, NY). Anti-Boss antibodies (anti-Boss) were provided by Larry Zipursky (University of California, Los Angeles). Anti-Bar antibodies were obtained from K. Saigo (Japan).

Drosophila opsin cDNA clones were obtained from C. Zuker (University of California, San Diego).

Light Microscopy

Tissue for light microscopy was prepared as follows. Fly heads were cut across the midline and immediately fixed for 4 h in 2% paraformaldehyde and 2% glutaraldehyde in 0.075 *M* Na cacodylate (PGC) buffer, pH 7.2. Subsequently the tissue was stained/fixed overnight at 4°C in 1% tannic acid in PGC. Samples were then washed (3 X 10 min in 100 *mM* Na cacodylate buffer, pH 7.2; 3 X 10 minutes in distilled water) and dehydrated via the following steps: 50, 70, 80, and 90% ethanol, 5 min each followed by 3 X 5 min in 100% ethanol. The tissue was then permeabilized in xylene / ethanol (1:1, 30 min) and pure xylene (30 min). For the embedding procedure, samples were incubated in 3: 1 and then 1: 1 xylene: PolyBed medium (PolyBed Media: 12.8 g of PolyBed 812 embedding media, 6.76 g dodecenylsuccinic anhydride, 5.46 g nadic methyl anhydride, and 0.5 mL of 2,4,6, tri- (dimethylaminomethyl) phenol; Polysciences, Inc.) and then incubated overnight at room temperature in 100% PolyBed media. Tissue samples were then placed in blocks containing fresh PolyBed and allowed to harden by incubating them at 35, 45, and 60°C over the course of 3 days. To follow the morphology of individual ommatidia through the depth of the retina, serial tangential sections (4 μm) were cut and viewed as follows Beginning with sections that were 16 μm beneath the surface of the retina, low magnifications of sections were projected onto a color monitor using video microscopy. The size, shape, and distribution of each ommatidium were

then recorded by tracing the image onto transparencies. Individual ommatidia were then visualized under phase microscopy (100-200X) and scored for the presence, number, and position of intact rhabdomeres. These data were recorded on the transparencies. By aligning the tracings of consecutive sections, individual ommatidia could be identified and scored at both the apical and basal regions of the retina. For an ommatidium to be scored positive, an R cell with an intact rhabdomere had to be observed in two or more adjacent sections. Ommatidia that were scored negative at one level, yet could not be judged at other levels due to the angle of the cut, were eliminated from consideration in this study.

Electron Microscopy

Tissues for electron microscopy were prepared as described earlier (Karpilow et al., 1989).

Isolation of Poly (A)+ RNA and Northern Analysis

Isolation of polyA$^+$ RNA from 1-to 2-day-old flies followed the procedures described by Zuker (Zuker et al., 1985). The gel electrophoresis and RNA transfer onto Nytran were done according to Sambrook *et. al.* (Sambrook et al., 1989) with the following modifications: the 10x gel-running buffer was 100 *mM* MOPS, 50 *mM* sodium acetate, pH 8.0, 10 *mM* EDT A, pH 8.0; and after the electrophoresis was completed, the 1.4% formaldehyde gel was soaked in 20x standard saline citrate (SSC) for 40 min prior to transfer to Nytran.Rhabdomere 1, 3, and 4 (Rh 1, Rh3, and Rh4) probes were generously donated by C. Zuker at the University of California,

San Diego. Fragments unique to each opsin were prepared (Montell et al., 1987; Zuker et al., 1985; Zuker et al., 1987) and labeled by nick translation. Hybridization and washing conditions were as described in (Sambrook et al., 1989). RNA hybridization bands were analyzed on the AMBIS Radioanalytic Imaging System and compared with levels of actin transcript to quantify opsin mRNA levels.

Immunohistochemistry and β-Galactosidase Staining in Enhancer Trap Lines

To visualize β-Galactosidase expression in the enhancer trap lines, eye-antennal imaginal discs were dissected from wandering third instar larval and stained as described by (Kramer et al., 1991). Mouse anti-β-Galactosidase antibody was obtained from Promega Biotech Inc. (Wisconsin). Goat anti-mouse antibody conjugated to horseradish peroxidase was obtained from Biorad Inc. (California). The anti-Boss antibody staining was according to procedures described by Kramer *et. al.* (Kramer et al., 1991). Eye discs were stained with anti-Sca antibodies according to (Baker et al., 1990). The procedure for the anti-Bar anti- body staining was as described by (Higashijima et al., 1992). Cell death was monitored in third instar eye discs by acridine orange staining as described by (Bonini et al., 1993).

Chapter III

Cell Cycle Regulation and Pattern Formation in the Developing Eye: *rap* regulates Mitotic Exit and Cellular Pattern Formation.

Summary

Abstract: In this chapter, my studies show that the *rap* (*retina aberrant in pattern*) gene encodes the Fizzy-related protein (Fzr), a protein with WD (Trp-Asp) repeat domains which plays an important role in regulating the mitotic exit. Abnormal additional mitotic cycles induced by loss-of- function mutations in *rap* lead to defective patterning of the developing *Drosophila* eye. Targeted over-expression of *rap/fzr* in the eye primordial cells causes precocious cell cycle exit, and smaller primordial eye fields, which either eliminate the eye or drastically reduce the size of the adult eye. Although mitosis is inhibited, abnormally large cells that form tumor like structures result from continued endoreplication, cell growth and retinal differentiation. Surprisingly, over-expression of *rap / fzr* in the eye primordial also increases the size of the antennal primordium and induces ectopic antennae. In sum, these results suggest that cell competition and inhibitory mechanisms may regulate the size of developmental fields during pattern formation and organogenesis, and that cell cycle exit is a hallmark for pattern formation and morphogenesis.

Introduction

During the development of multicellular organisms, cellular pattern formation is coordinated with mechanisms that regulate cell proliferation, growth and death. The development and correct patterning of adult structures requires that appropriate numbers of undifferentiated cells be generated at specific developmental stages. Once the correct number of precursors is generated, the cells must exit the cell cycle to ensure proper cell fate specification and pattern formation. Therefore progenitor cells must continuously enter into cell cycles and exit at the appropriate time. When a cell enters the cell cycle it follows a number of orderly and unidirectional biochemical transitions that will culminate in the creation of two cells with identical amounts of genetic material. The mitotic cell cycle consists of two major events: replication of the genetic material and separation of the chromosomes. These two actions are biochemically distinct and are separated by a series of transitional steps. The transition of every step in the cell cycle is regulated by a series of intrinsic and extrinsic mechanisms (Elledge, 1996). The intrinsic mechanisms ensure that the transitions take place in an ordered and unidirectional fashion. Extrinsic mechanisms are activated only in the event that any defect in DNA replication or chromatid separation occurs (Elledge, 1996). These intrinsic or extrinsic transitional mechanisms are called checkpoints (Hartwell and Weinert, 1989).

A Brief Review of Cell Cycle Regulation

Enzymes known as cyclin-dependent kinases (Cdks) are the key regulators of the cell cycle. In turn, Cdks are regulated by proteins called cyclins which are required for the kinase activity of the Cdk as well as for the substrate specificity of the Cyclin/Cdk complex (Koepp et al., 1999). Once activated, the Cyclin/Cdk complex operates by phosphorylation of proteins that are involved in the regulation of DNA replication, chromosome segregation, and cytokinesis (Murray and Hunt, 1993). Sequential activation of the right Cdk with its respective cyclin will trigger the onset of the S phase or M phase of the cell cycle (Figure 16). Cyclins and Cdks appear to be conserved among multicellular eukaryotes (Edgar and Lehner, 1996). In *Drosophila* and vertebrates, cyclins type A, B, D and E and Cdk1, Cdk2, Cdk4 and Cdk6 have been found to have roles in cell cycle control (Edgar and Lehner, 1996). Cyclin D regulates progression through the G1 phase of the cell cycle in complex with Cdk4 or Cdk6. Cyclin E/Cdk2 regulates the entry into the S phase. Overexpression of Cyclin D and Cyclin E will force the cell to enter the S phase precociously (Ohtsubo and Roberts, 1993; Quelle et al., 1993). CyclinA/Cdk2 regulate progression through S phase. Cyclin A and B in complexes with Cdk1 regulate entry into the M phase (Sherr, 1994).

Although each phase of mitosis requires a specific Cyclin/Cdk complex which allows the cell to proceed through that phase, the Cyclin/Cdk complex must be inactivated once the cell completes that particular phase. Frequently such negative regulation is controlled by ubiquitin-mediated proteolysis (Koepp et al., 1999).

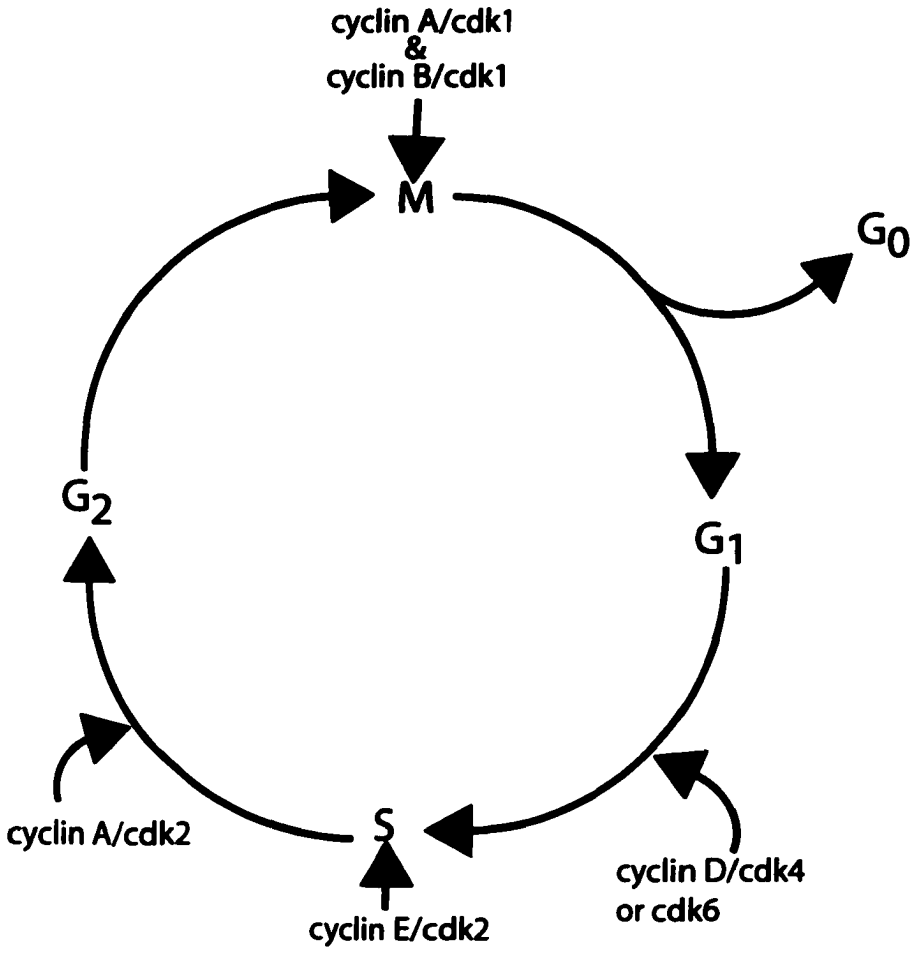


Figure 16. Cyclin/cdk complexes regulate the transitions of the cell cycle. Cyclin E/Cdk2 complex regulates the entry into the S phase. CyclinA/Cdk2 regulate progression through S phase. Cyclin A/Cdk1 and cyclin B/Cdk1 regulate the entry into the M phase. Cyclin D regulates progression through the G1 phase of the cell cycle in complex with Cdk4 or Cdk6.

The ubiquitination of cyclin or Cdks can be divided into two categories: the ubiquitination that is required for cell cycle progression, e.g. destruction of cyclin B, and the ubiquitination that is important but is not essential for cellular homoeostasis, e.g. G1 cyclins (Koepp et al., 1999).

In higher eukaryotes, two ubiquitination complexes, the APC (anaphase promoting complex) and the SCF (Skp-cullin-F box protein), each composed of about 12 proteins regulate cell cycle progression by the timely degradation of key mitotic regulators such as cyclins and cyclin dependent kinases. The targeting of the cyclins to the APC and SCF complexes is mediated by proteins containing CDC 20 or the 'Fizzy' domain, which contains the conserved WD repeat motif. It has been proposed that the WD and F-boxes are required for the recruitment of substrate to the SCF complex (Skowyra et al., 1997).

WD Domain Proteins and Cell Cycle

Proteins with the WD domains (also known as WD-40) are found only in eukaryotes and this domain is repeated four to eight times within each polypeptide. The repeated motif is called the WD domain because this domain usually ends with the sequence Trp-Asp (WD) (Neer et al., 1994). These WD repeats were initially found in the β-subunits of heterotrimeric GTP-binding protein (G-protein), which transduce signals across the plasma membrane (Fong et al., 1986). They have been called the β-transducin repeat (Duronio et al., 1992), the WD 40 repeats (Fong et al., 1986), or the GH-WD repeat (Neer et al., 1993). These repeats (Figure 17) have a region of variable length, which is followed by a core of a more or less constant region flanked by two characteristic pattern elements of GH (Gly-His) and WD.

Eighty two percent of the repeating units contain a maximum range of 23-41 amino acids between GH and WD. There are 36-46 WD amino acid long units in the WD domain (Neer et al., 1994).

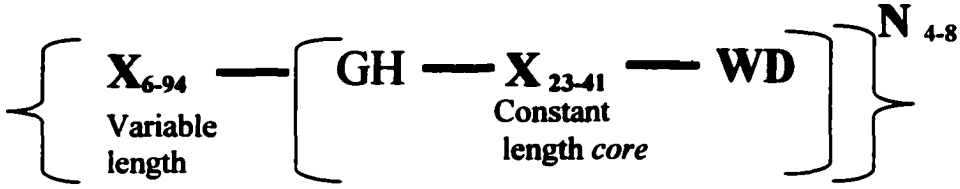


Figure 17. General pattern of the prototype of the WD repeat. The WD repeats have a region of variable length followed by a core of more or less constant region which is flanked by a region comprised of Gly-His on one side and the WD region on the other (Neer et al., 1994).

All the WD proteins that have been characterized so far have a regulatory function and none of them have been shown to have an enzymatic role. The WD proteins have a wide variety of functions, including assembly of macromolecules for mRNA splicing (Banroques and Abelson, 1989; Bjorn et al., 1989), mRNA modification (Takagaki and Manley, 1992), and vesicular traffic (Harrison-Lavoie et al., 1993; Stenbeck et al., 1993). The wide variety of function suggests that these repetitive units may have become functionally specialized (Neer et al., 1994).

Even though initially these proteins were thought to function specifically in cell cycle regulation, recent work has shown that several of the SCF and APC activating proteins are expressed in post-mitotic and differentiated cells (Gieffers et al., 1999). The function of Fizzy domain proteins in postmitotic cells or other differentiated cells is not clear. One possibility is that they are required in postmitotic

cells to prevent premature or inappropriate entry into S phase and thus play a role in maintaining the differentiated state. A second interesting possibility is that in addition to their role in cell cycle control these Fizzy domain proteins may target other important cellular proteins to the APC complex for ubiquitination and thus regulate other cellular functions. There are other examples of proteins that, in addition to functioning as negative regulators of cell cycle, are also involved in patterning and cell differentiation.

Ebi

The GTP-binding protein Ebi, contains a WD domain and an F-box motif, characteristic of proteins of the SCF complex. Ebi promotes G1 arrest in the developing eye. Ebi initially was shown to be involved in retinal patterning through its interaction with the EGFR signaling pathway (Dong et al., 1999). *ebi* mutant embryos posses many of the phenotypes previously described for *egfr* mutant embryos (Dong et al., 1999). Ebi has also been shown to promote neuronal differentiation by down-regulating Tramtrack88 (TTK88) in the eye imaginal disc (Dong et al., 1999). TTK88 is an ETS-domain transcription factor that functions to repress genes required for photoreceptor differentiation. Studies of *ebi* in the compound eye revealed that it is required for normal differentiation of the R7 cell. It is possible that Ebi serves to coordinate cell cycle exit with the start of cell differentiation (Boulton et al., 2000).

Roughex:

Similarly, other negative regulators of cell cycle have been characterized. Cyclin dependent kinase inhibitors bind (CdkI) to the Cdk complexes and inhibit their enzymatic activity (Cunningham and Roussel, 2001; Lee and Yang, 2001; Toogood, 2001; Yew, 2001). Roughex (Rux) is a CdkI that acts as a negative regulator of cyclin A by promoting its nuclear localization and thereby its rapid degradation in G_1 (Thomas et al., 1997). Although Rux binds cyclin E, it does not inhibit the activity of the Cyclin E/Cdk (Thomas et al., 1997). In late G_1, cyclin E destabilizes Rux resulting in the release of cyclin A. Once free, cyclin A performs its role in S/G2. Mutations in the *roughex* locus result in the accumulation of cyclin A in G_1 causing cells to enter S phase precociously (Thomas et al., 1997).

Dacapo:

Another negative regulator of the cell cycle is Dacapo (Dap). This protein is a member of the p21/p27 family of CdkI. Dacapo is required for the timely exit from the cell cycle. This protein interacts genetically with the retinoblastoma homolog (Rbf) and cyclin E/cdk2 complexes. During development, *dap* mRNA expression coincides with cell cycle arrest and overexpression of *dap* inhibits cell cycle progression in vivo (de Nooij et al., 2000). It is interesting that *dacapo* mRNA and protein levels are dependent on Cyclin E expression. Thus, expression of Cyclin E induces *dacapo* expression (de Nooij et al., 2000). Continuous expression of Cyclin E can force cells to commit to an additional round of cell cycle (Knoblich et al., 1994). Thus, the expression of Cyclin E will trigger transcription of *dacapo* which will negatively regulate Cyclin E thus, arresting the cells in G_1.

Archipelago

Archipelago (ago) is another negative regulator of Cyclin E (Moberg et al., 2001). Genetic and physical interaction studies show that *ago* physically interacts with Cyclin E (Moberg et al., 2001). *ago* encodes a WD-domain protein that contains an F-box, which functions as the substrate-recognition component of the SCF-type ubiquitin ligase complex (Bai et al., 1996; Moberg et al., 2001; Skowyra et al., 1997). Mutations in the WD domain of Ago prevent its binding to phosphorylated Cyclin E and thus elevate the levels of this mitotic cyclin and prevent cells from entering G_1 arrest. In *ago* mutant tissue, an increase in the number of cells that enter S and G2/M phases is observed, as well as a decrease in the number of cells that are arrested in G_1 (Moberg et al., 2001). This pattern is correlated with the effect of overexpression of Cyclin E (Neufeld et al., 1998). It was concluded that Ago regulates the levels of Cyclin E post-transcriptionally during normal development (Moberg et al., 2001).

Fizzy:

The Fizzy (Fzy) protein contains WD repeats in its C-terminal domain and is required for normal execution of the metaphase-anaphase transition and mitotic cyclin degradation (Dawson et al., 1993; Dawson et al., 1995; Lorca et al., 1998; Sigrist et al., 1995). Its sequence revealed that the carboxyl half of this protein has significant homology to the *Saccharomyces cerevisiae* cell cycle gene CDC20 (Dawson et al., 1995). Fzy is part of a high-molecular-weight complex distinct from the APC/cyclosome. However, Fzy is required for APC/cyclosome activation, for the

degradation of mitotic cyclins A, cyclin B, cyclin B3 and for proper chromatid segregation in the cell cycle extracts prepared from Xenopus eggs (Lorca et al., 1998). Mutations in *Drosophila fzy* result in metaphase arrest in which neither Cyclin A, Cyclin B or Cyclin B3 are degraded (Dawson et al., 1993; Dawson et al., 1995; Lorca et al., 1998). Thus, Fzy is required for the segregation of sister chromosomes to the spindle poles and for sister chromosome separation (Lorca et al., 1998).

Cell cycle events in the developing eye

The compound eye of *Drosophila* has emerged as an attractive system to study the relationship between cell cycle control, cell differentiation and pattern formation. Two waves of mitosis occur during pattern formation in the developing eye disc (Figure 18) (Wolff and Ready, 1991). The first wave is asynchronous and occurs anterior to the morphogenetic furrow (MF). A region of G1 arrest in the MF follows this active mitotic region. The second mitotic wave occurs immediately posterior to the MF, it is synchronized and appears as a compact band (Wolff and Ready, 1991). Other laboratories have characterized the domains of expression of various genes and mitotic events in the developing eye imaginal disc (Penton et al., 1997; Thomas et al., 1994). The anterior region of the eye disc ahead of the MF can be divided into two domains. In the first domain expression of Cyclin B and Cyclin A can be detected by antibody staining. These cyclins start to be expressed during S phase and G_2 to regulate the G_2 to mitosis transition (Sherr, 1994) (Figure 18).

Labeling with Bromo-deoxyuridine (BrdU), which labels cells in the S phase, reveals that another subgroup of cells of this area are in the S phase of the cell cycle.

Staining with propidium iodide, to label cells going through M phase, can be detected in this region. Thus, the first domain ahead of the MF contains cells in various stages of the cells cycles (Figure 18).

The second domain of the anterior region of the eye disc is marked by the expression of *string* mRNA. String is a phosphatase homologous to and the functional equivalent of mitotic inducer Cdc25 (Edgar and O'Farrell, 1989; Kumagai and Dunphy, 1991). The *string* mRNA is expressed in 5-6 columns of cells just ahead of the MF (Alphey et al., 1992). In the region where string is expressed no S-phase cells are detected with the BrdU incorporation assay. Cells stop incorporating BrdU in the anterior boundary of the expression domain of string. Analysis of the 5-6 columns of cells just anterior to the MF with propidium iodide reveals that a large number of cells are going through mitosis in this region. These could be cells that were already committed to the cell cycle in the previous domain and are just finishing the cycle prior to the MF (Figure 18).

Cells in the MF are arrested in G_1 phase of the cell cycle. In this domain no staining of Cyclin A, Cycling B, BrdU or propidium iodide is observed (Thomas et al., 1994). The second mitotic wave (SMW) is seen immediately posterior to the MF and is characterized by the onset of BrdU incorporation followed by the expression of Cyclin A, and Cyclin B as well as staining with propidium iodide (Thomas et al., 1994). Genes that play a role in the regulation of these events have been studied in some detail (Penton et al., 1997). The differentiated cells just behind the MF express the Hedgehog (Hh) protein, which activates transcription of the transforming-growth-factor β-related gene *decapentaplegic* (*dpp*) in the MF. DPP is required in the MF for

cell cycle synchronization and G1 arrest prior to the assembly of ommatidia (Penton et al., 1997). DPP diffuses anteriorly, inducing cells to enter the M phase ahead of the MF. Possibly DPP accomplishes this job by regulating the transcription or activity of genes that are involved in the G_2-M transition (Penton et al., 1997). Rux prevents cells from entering the S phase prematurely (Thomas et al., 1994).

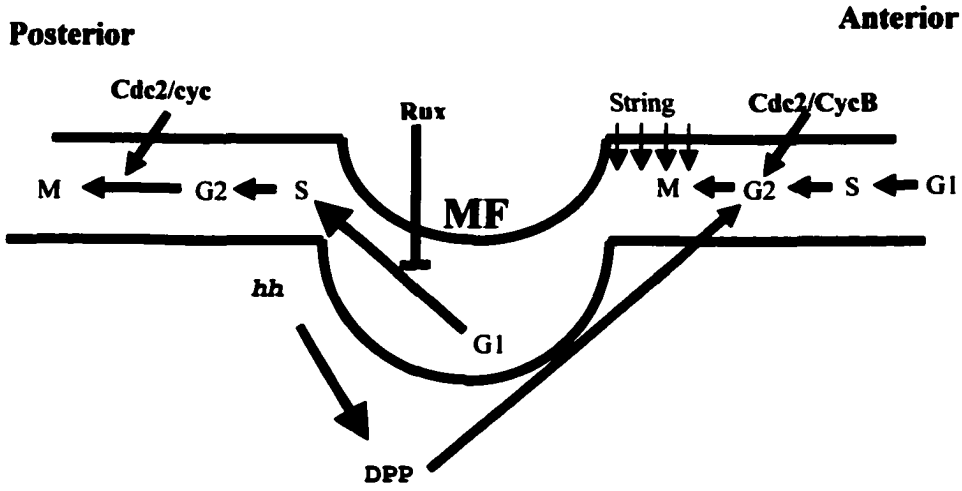


Figure 18. Diagram of the different cell cycle domains in the developing third instar eye imaginal disc. In the anterior part of the developing eye disc, expression of CycB/Cdc2 and CycA/Cdc2 can be observed. These two Cyclin/Cdk complexes promote the transition from G2 to M. Just anterior to the MF 5-6 columns of cells express the mitotic inducer string. The anterior boundary of the string expression, cells stop going into S phase and only those cells already committed to another round of the cell cycle finish it within this boundary. In the MF cells, are arrested in the G1 phase presumably by the action of the Rux protein. Posterior to the MF undifferentiated cells will initiate another round of mitosis and expression of CycB/Cdc2 and CycA/Cdc2 can be seen again. In the posterior part of the eye disc differentiated cells express the Hedgehog (Hh) protein, which activates transcription of *dpp*. Dpp is required for cell cycle synchronization and G1 arrest in the MF Figure modified from Thomas et al, 1994 and Penton et al.

Results

Rap regulates cell cycle in the developing eye

To ascertain whether the aberrant patterning in the *rap* mutants was due to defects in cell cycle regulation, I examined the mitotic pattern in wild type and loss-of-function *rap* mutants. Cellular pattern formation in the eye imaginal disc is marked by the anterior progression of the Morphogenetic Furrow (MF). Pattern formation and cell fate determination in the developing eye are precisely coordinated with a stereotypical pattern of two mitotic cell cycles. In the wild type (normal) developing eye, the first mitotic domain is seen anterior to the MF where cells divide asynchronously and form a diffuse band of mitosis as seen by BrdU incorporation (Figure 19 A). Immediately posterior to the MF, the second mitotic wave (SMW) is seen as a compact band of S phase cells labeled with BrdU (Figure 19 A). BrdU labeling of third instar *rap* mutant eye discs showed that the second mitotic wave was disorganized and mis-regulated in *rap* mutants (Figure 19 B). S phase cells were seen within the MF, where normally cells are arrested in G1. Similarly, cells in posterior regions of the disc, which are normally post-mitotic, also showed BrdU incorporation indicative of a large number of S phase cells. These results suggest that in the *rap* mutant, cells fail to arrest in the G1 and cells posterior to the MF, which are normally post-mitotic, were initiating aberrant additional rounds of the cell cycle.

Figure 19. Rap regulates mitosis: To follow the pattern of mitosis in the developing compound eye, wild type and *rap* mutant developing eye discs were labeled with Bromdeoxyuridine (Brdu). In the wild type (A), two characteristic bands of Brdu labeling are seen Anterior to the morphogenetic furrow (Arrow heads, MF), Brdu labeling appears as a broad band, whereas, posterior to the MF, Brdu labeling is in a compact band of cells. The differentiated regions posterior to the MF show no Brdu labeling. In the rap^{X3} mutant discs (B), Brdu labeling is diffusely distributed over the entire disc including the regions posterior to the MF and the organization of the two distinct domains seen in the wild type is lost. In *rap* mutants, cells fail to exit the mitotic cycle and go through additional mitosis. (C) Wild type eye disc stained with anti-cyclin B (green) and Anti-phospho Histone-H3 (red) shows that the mitotic marker Histone H3 and cyclin B are localized to regions anterior to the Morphogenetic furrow (MF) and immediately posterior to it. No cyclin B is seen in the MF. (D) rap^{X3} mutant eye disc stained with anti-cyclin B (green) and anti-H3 (red) shows uniform distribution of cyclin B and persists in the MF. Significantly large numbers of H3 positive cells are seen in the normally post-mitotic regions posterior to the MF. (E) A *dpp-lacZ*, eye disc stained with anti-cyclin B (green), Anti-elav (red) and anti β-gal for *dpp* (blue) shows that the G2 Cyclin B is restricted to the mitotic areas and is not seen in the MF. In panel (F), eye imaginal disc from *dpp-LacZ/+;* rap^{X3}/y larva stained with anti-cyclin B (green), anti-elav (red) and anti β-gal for *dpp* expression (blue), shows cyclin B is distributed uniformly throughout the disc including the MF and posterior region of the disc where the cells are post-mitotic in the wild type. In panels E and F the morphogen DPP is expressed in the morphogenetic furrow and promotes G1 arrest. Anterior is to the right.

To determine if these cells had progressed from the G_1/S to the G_2/M stage, I next studied the distribution of cyclin B, a marker for the G_2 stage of the cell cycle. In the wild type developing eye disc, cyclin B distribution overlaps the BrdU labeling pattern. Cyclin B is distributed uniformly in the regions anterior to the MF and is degraded within the MF where cells are arrested in G_1. Cyclin B is also seen in a narrow band immediately posterior to the MF overlapping the BrdU label as a result of the second mitotic wave and cyclin B is not seen in the posterior regions of the disc where cells are post-mitotic (Figure 19 C).

In the *rap* mutant eye disc, cyclin B persists in the MF and in the post-mitotic regions posterior to the second mitotic wave (Figure 19 D). To localize mitotic phase (M) cells in the developing eye imaginal discs, I labeled imaginal discs with antibodies against human Phospho-Histone H3, a marker for cells in the mitotic phase of the cell cycle (Wei et al., 1999). In wild type eye imaginal discs (Figure 19 C), two distinct domains of mitotic nuclei (corresponding to the two zones of BrdU labeling) are seen. Anterior to the MF, M phase cells are distributed diffusely. Immediately posterior to the MF, a narrow band of Phospho-H3 labeled cells localize corresponding to the second mitotic wave. In contrast, in *rap* mutant eye discs, the entire disc posterior to the MF contains a large number of M phase cells with no evidence of a narrow band of cells undergoing a discreet second wave of mitosis. The number of M phase cells anterior to the MF is comparable to the wild type discs (Figure 19 D).

To follow neuronal differentiation in the developing eye imaginal disc, I next localized the neuron specific marker Elav using anti-Elav antibodies. My results

show that in *rap* mutants, while spacing and size of the ommatidial clusters was abnormal, neuronal cell differentiation was not affected (Figure 19 F). I also monitored *dpp* expression in the MF of developing eye imaginal discs using larval carrying a *dpp-lacZ* construct. My results showed that *dpp* expression in *rap* mutant discs was similar to that in the wild type discs (Figure 19 F), suggesting that *rap* may function downstream of *dpp*. Taken together, these results suggest that the wild type function of *rap* is necessary for the timely degradation of cyclin B and timely mitotic exit of the precursors cells in the developing eye disc. These data support the idea that, in loss-of function *rap* mutants, cells posterior to the MF fail to exit the mitotic cell cycle at the appropriate time and initiate additional abnormal cell cycles. These results are consistent with results in the embryo reported by Sigrist and Lehner (1997).

Rap *encodes the Fizzy related protein (Fzr)*

Isolation of new alleles of *rap*

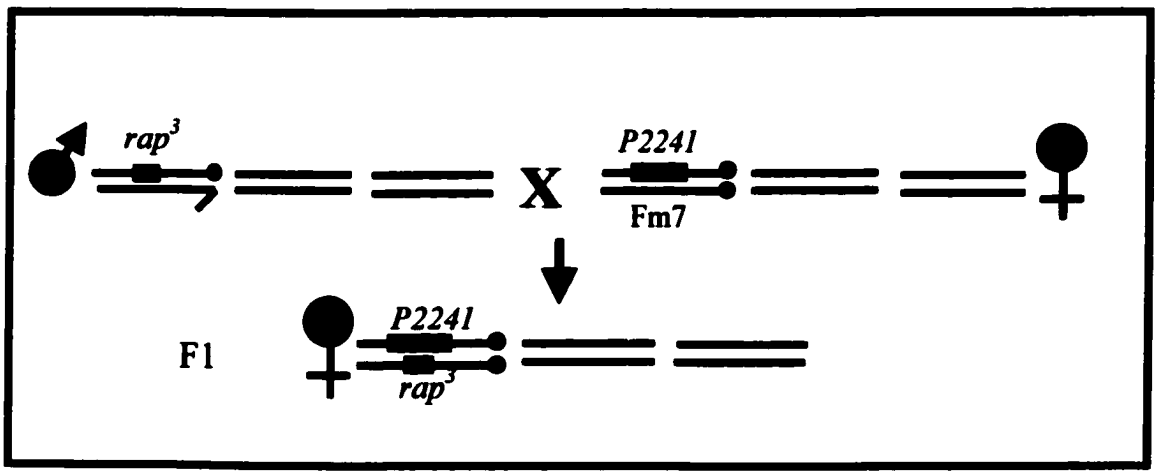
As a first step toward molecular cloning, and to better definition of the *rap* locus, three new alleles were isolated using X-radiation and EMS mutagenesis (Table 2). Work from our laboratory had previously mapped the *rap* locus to the 4C7-4C15 region of the X chromosome by recombination and deficiency mapping (Karpilow et al., 1989). In order to clone the *rap* gene, I searched the *Drosophila* genome project database for P insertions in the 4C7 region, with the expectation that some of the insertions may reside in the *rap* locus. The P insertions were tested for complementation with *rap* alleles, with the idea that, if the P insertion disrupts the *rap*

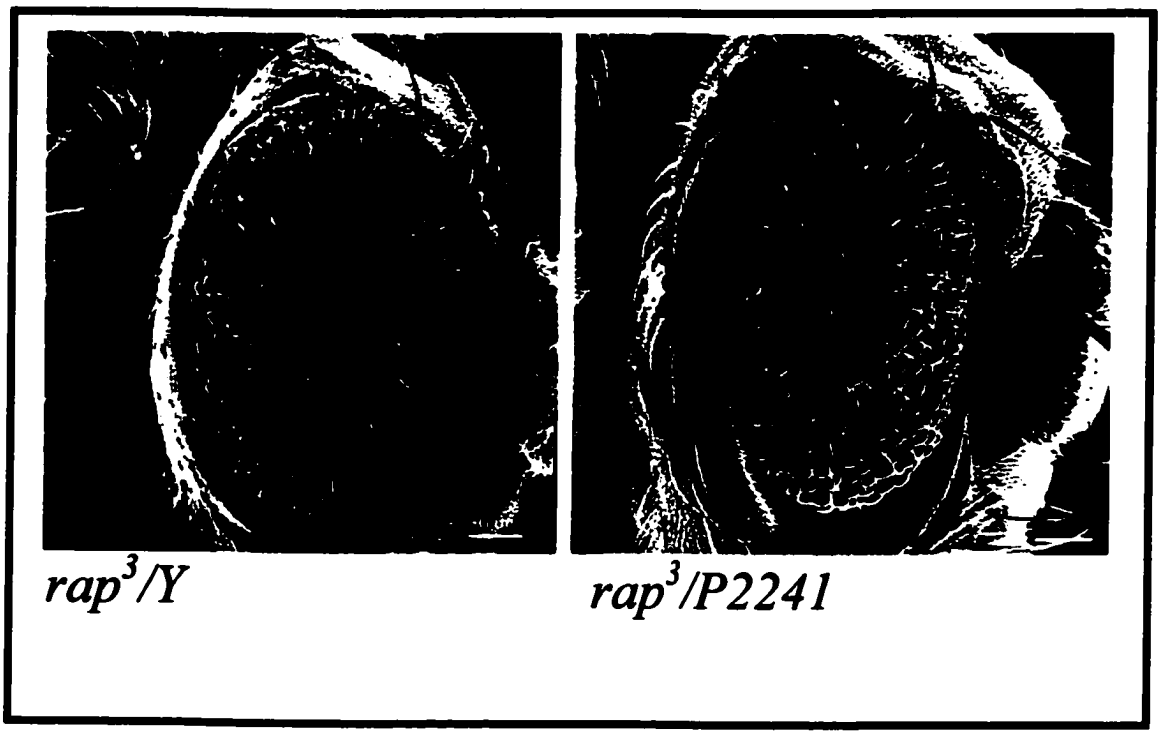
gene, then the insertion would fail to complement the *rap* rough eye mutant phenotype. On the other hand complementation would indicate that the P-insertion was not in the *rap* locus. This is with the caveat that *rap* is a simple locus. Several P insertions were tested and one, the P-2241, failed to complement the *rap* alleles suggesting that the P element was inserted in the *rap* gene (Figure 20). Every allele of *rap* was tested for complementation with P-2241.

Allele	Mutagenic Agent	Phenotype	Classification*
rap^{1}	P-element	++ Weak	Hypomorph
rap^{2}	P-element	+++ Moderate	Hypomorph
rap^{3}	P-element	++++ Strong	Hypomorph
rap^{x2}	X-rays	+++ Moderate	Hypomorph
rap^{x3}	X-rays	++++ Strong	Null,
$rap^{E\text{-}L3}$	Ethylmethane Sulfonate (EMS)	Lethal	Null-Lethal
rap^{E1}	EMS	+++ Moderate	Hypomorph
rap^{E4}	EMS	+++ Moderate	Hypomorph
rap^{E6}	EMS	+++ Moderate	Hypomorph
rap^{P2241}	P-element	Lethal	Null

Table 2. List of *rap* alleles isolated and their phenotypes.
*** classification based on genetic criteria.**

Figure 20. The P lethal stock 2241 fails to complement the *rap* phenotype. The top panel shows the complementation cross. The bottom panel show in A) the scanning electron micrograph of rap^3/Y. B) Scanning electron micrograph of rap^3/*P2241*, which fails to complement the *rap3* locus. This result indicates that the P element of *P2241* is inserted in the *rap* locus.





In order to clone the DNA sequences flanking the P insertion I used inverse PCR technique (Ochman et al., 1998). DNA fragments from the inverse PCR product was sequenced and analyzed by the BLAST program. BLAST analysis revealed that the P-element was inserted into the first intron of the *fizzy related* (*fzr*) gene (see appendix 2) (Sigrist and Lehner, 1997). *fizzy related* gene encodes a 478 amino acid protein with seven WD-40 domains. *fzr* negatively regulates the levels of cyclins B, B3 and A, during the G1 stage of the cell cycle (Sigrist and Lehner, 1997). These mitotic cyclins activate Cdk1 (cdc2). *fzr* has homologous in human (*hcdh1*), *S. cerevisiae* (*srw1p*), and Xenopus and it is a regulator of the APC complex (Kominami et al., 1998; Kramer et al., 1998; Yamaguchi et al., 2000).

In addition to cloning the *rap* gene using the inverse PCR technique, I also cloned the DNA flanking the P insertion 2241 using the plasmid rescue technique. The genomic DNA from P2241 flies were isolated, and plasmid rescue was done as previously described (Guo et al., 1996; Hersberger et al., 1996) digested with EcoRI and Xba1 enzymes and ligated. DNA was PCR amplified and its sequence determined. Sequence comparison to homologous sequences by BLAST analysis showed that the P insertion was within the first intron of the *fzr* gene (Figure 21). These results are consistent with the results obtained from the inverse PCR experiments.

Southern analysis of the *rap* alleles shows that *fzr* is derived from the *rap* locus.

The rationale for these experiments was that if *rap* encoded Fzr, analysis of the *rap* mutant DNA on Southern blots with *fzr* as a probe would reveal restriction

fragment length polymorphism (RFLPs) due to the mutational lesions. To study this, I probed the genomic DNA from all *rap* alleles with *fzr* cDNA. Genomic DNA from *Canton-S* (*CS*) and *Oregon-R* (*OR*), *4A2A* (parental strains) and the various *rap* alleles were isolated, digested with HaeIII restriction enzyme, and fractionated in an agarose gel. These samples were transferred to a nylon membrane and a 2.6Kb *fzr* cDNA (Sigrist and Lehner, 1997) were used to probe the membrane as the probe (Figure 22). The *rap* mutant alleles rap^{X2}, rap^{X3} were obtained by mutagenizing CS flies with gamma ray (Ashburner, 1989). The rap^{E2}, rap^{E4} and rap^{E6} alleles were obtained using EMS mutagenesis of the CS flies (Ashburner, 1989). The alleles *y,sc,*rap^{1}*,v,f* and $wrap^{3}$ were originally isolated by P-M hybrid dysgenesis (Karpilow et al., 1989) cross using the parental stock 4A2A. The 4A2A stock was originally generated from an Oregon-R (OR) stock. The Southern blot analysis (Figure 22) showed that parental strains; CS, OR and 4A2A have three DNA fragment of identical molecular weight that range between 1.0Kb and 0.75 Kb (Figure 22). The parental lines also show an additional 1.4 Kb band (arrow) in all alleles (black arrow Figure 22). With the exception of $wrap^{3}$, the *rap* mutant alleles show RLFPs. The DNA is from the alleles rap^{1}, rap^{X2}, rap^{X3}, rap^{E2}, rap^{E4} and rap^{E6}.

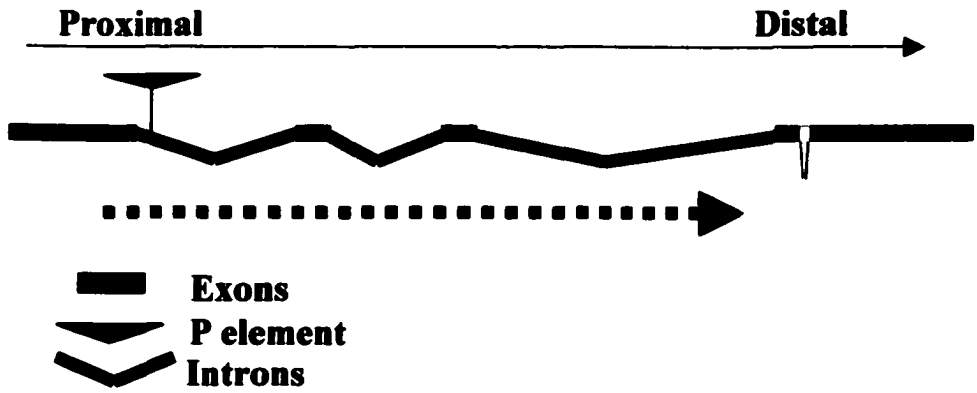
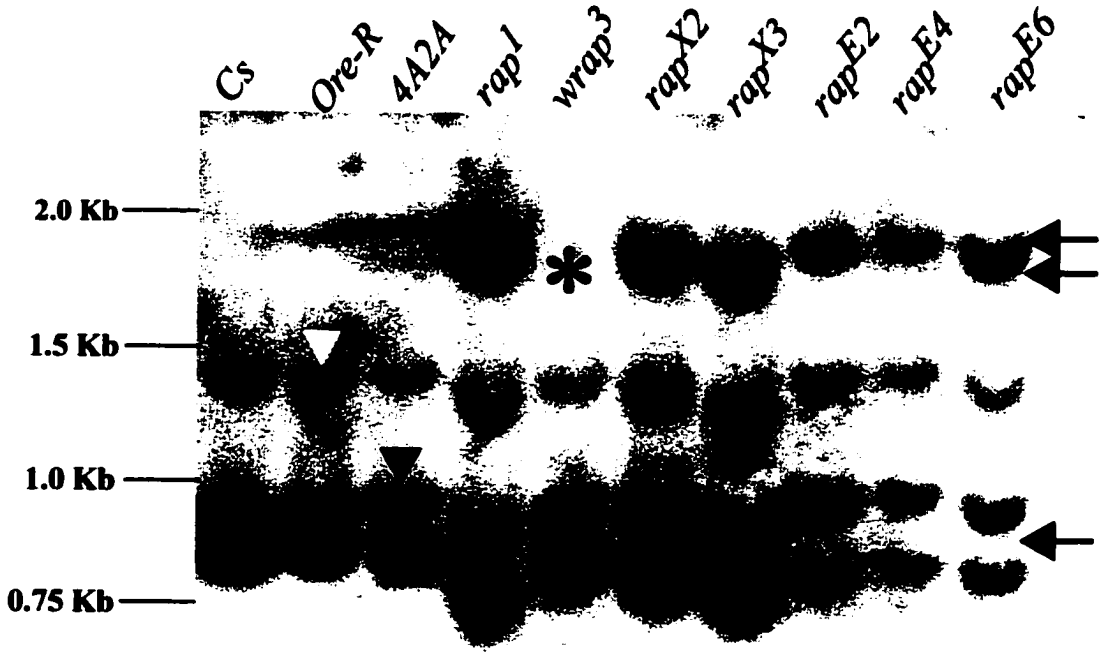


Figure 21. Molecular organization of the *rap/fzr* locus. The *rap/fzr* locus is comprised of five exons and four introns. The P element P2241 is inserted in the first intron of the *rap/fzr* locus. The intron-exon organization of the *rap/fzr* locus was derived from the *Drosophila* genome sequence annotation (www.fruitfly.org)

Figure 22. Southern blot analysis of *rap* mutants.
The 2.6 Kb *fzr* cDNA was radioactively labeled and hybridized to a genomic Southern blot that contained genomic DNA from *rap* mutant alleles and parental strains. The genomic DNA was digested with *HaeIII*. *CS*, *Ore-R* and *4A2A* were used as controls. A 1.4 Kb band (A, arrow head), is seen followed by three closed bands between 1.0 and 0.75 Kb (A, black arrow head). In all but $wrap^{3}$ (asterisk) the *rap* mutant alleles RFLPs are observed. In the *rap* mutants; rap^{1}, rap^{X2}, rap^{X3}, rap^{E2}, rap^{E4}, rap^{E6}, only two bands are seen (arrow), however unlike the wild type an additional band of 1.9 Kb is observed in *rap* mutants (double arrow) an additional band is seen at 1.9 Kb (white arrows and put lane number to each allele Figure 22). In the same alleles only two bands are seen in the 1.0 Kb to 0.75 Kb range. These results show that when probed with *fzr* cDNA the genomic DNA from the *rap* alleles shows RLFPs, which is consistent with the idea that *fzr* cDNA is derived from the *rap* locus.

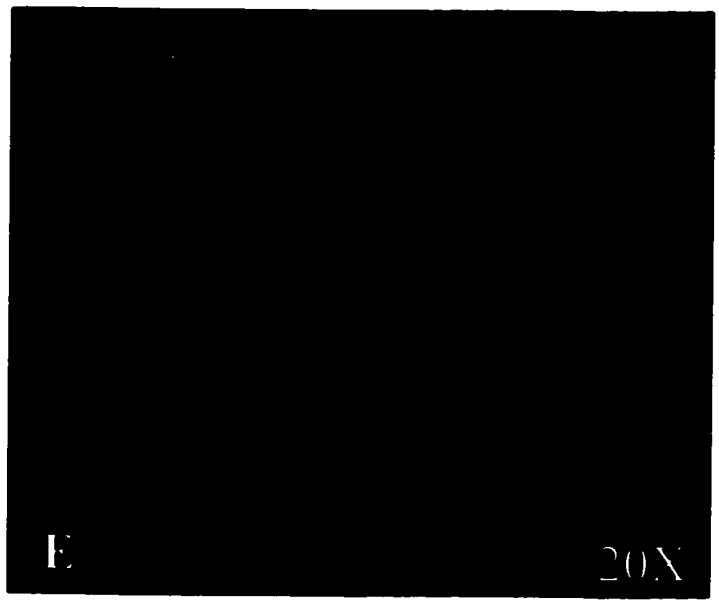


Transgenic expression of Fzr rescues *rap* mutant eye phenotype:

To obtain definitive evidence that the *rap* gene encodes the Fzr protein, I conducted germline rescue experiments. I expressed a full-length *fzr* cDNA transgenically using the binary GAL4-UAS expression system (appendix 3) (Brand and Perrimon, 1993). The GAL4-UAS system is a two-component system, for targeting tissue and cell type specific expression of genes of interest in *Drosophila*. The *fzr*-cDNA expression under the control of UAS sequences was driven by the *linotte (derailed)* GAL4 driver (fly base stock #4669). The *linotte* gene is allelic to *derailed* and is required for nervous system development and learning (Bolwig et al., 1995; Dura et al., 1993; Dura et al., 1995). The *rap* rough eye phenotype was completely rescued by the expression of Fzr (Figure 23)*. These data show that Fzr expression is sufficient to complement *rap* gene function. Taken together these results show that the *rap* gene encodes the Fzr protein.

* 67% Of the flies scored, showed complete rescue (Figure 23 D); 15% showed partial rescue (Figure 23 C). The remaining flies showed rescue to various degree. N=71

Figure 23. Transgenic expression of the *fzr* cDNA rescue the *rap* eye phenotype. The diagram on top shows an outline of the genetic cross of *linotte-GAL4* and rap^{X3}, *UAS-fzr*. In this cross, the expression of the *UAS-fzr* construct was driven under the *linotte-GAL4* driver. Scanning electron micrograph (SEM) of wild type compound eye (A), and rap^{X3}, *UAS-fzr* (B). Most of the progeny were partially rescued (C), while other group showed full rescue (D). To test the spatial expression of the *linotte* protein, the *UAS -β-galactosidase* construct was crossed to the linotte GAL4. The third instar eye imaginal disc from the F1 were dissected and stained with anti-β-galactosidase antibody. The result is shown in (E), the expression of linotte occurs in the posterior part of the eye disc and in the MF.



Expression of *rap/fzr* mRNA.

To determine the spatial pattern of expression of the *fzr* gene in the developing eye disc, I performed in-situ hybridization experiments in the *fzr* cDNA with third larval instar eye imaginal discs. Digoxigenin single stranded RNA probes were synthesized from the *fzr* cDNA using the RNA Labeling Kit from Roche. To record the expression of *fzr* the antisense strand was used. The sense strand RNA was used as a control. The antisense *fzr* RNA shows hybridization in the morphogenetic furrow and in the posterior part of the eye disc (Figure 24). There is no expression of *fzr* mRNA ahead of the morphogenetic furrow, and immediately behind it. In the most posterior part of the eye disc the transcription of *fzr* mRNA could be seen in the cell clusters. The morphogenetic furrow appears to have stronger expression of the *fzr* mRNA when compared with the expression in the posterior part of the eye disc.

Rap/Fzr Protein expression in the third instar eye imaginal disc.

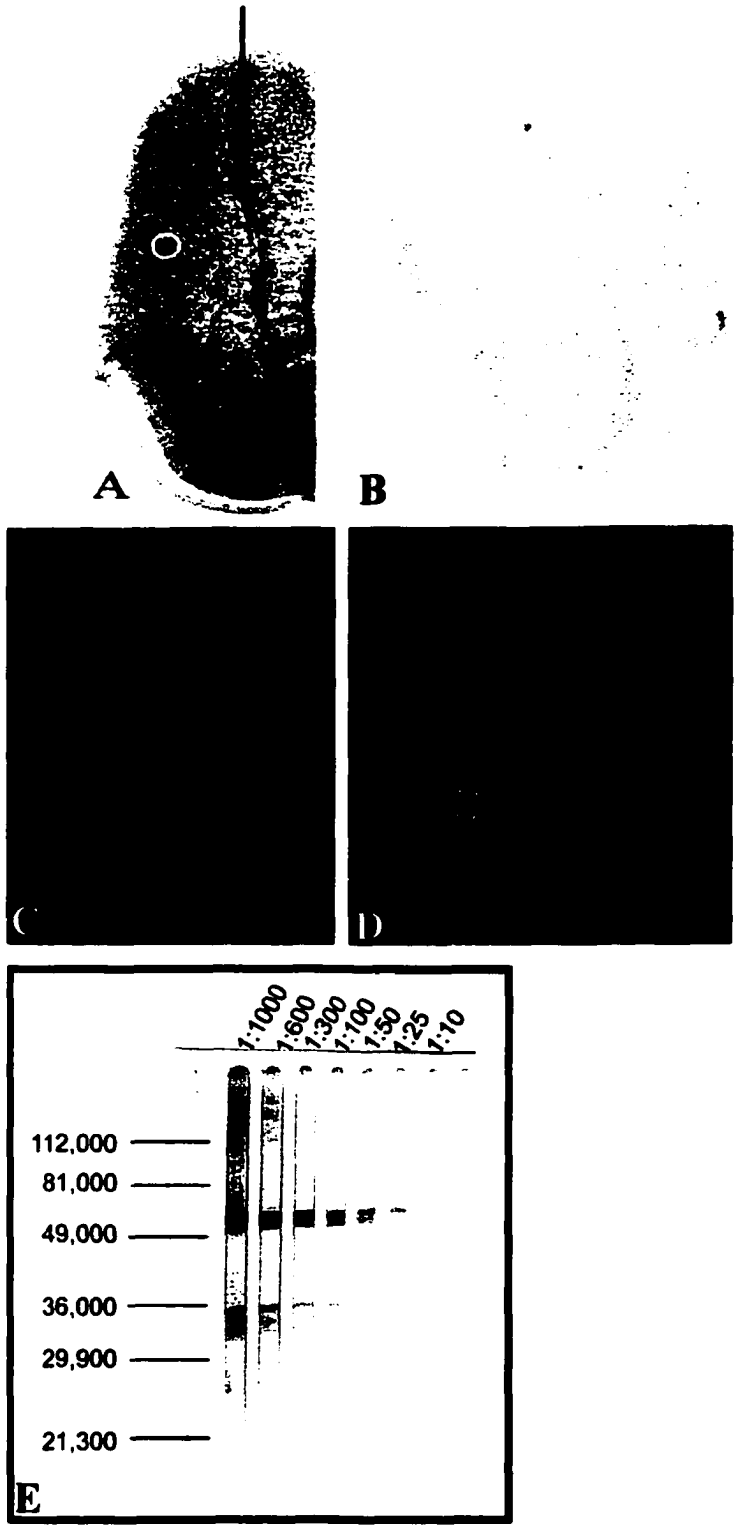
Using the published amino acids sequence from Fzr a synthetic oligopeptide was constructed and used to generate polyclonal antibodies in rabbit. Since Rap (Fzr) sequence is highly conserved from yeast to humans, I selected the amino acid sequence unique to *Drosophila* and synthesized peptides (see Table 3). These peptides were used to immunize rabbits and the anti Rap (Fzr) specific antibodies were affinity purified using an affinity column with the synthetic peptides as the affinity ligands. The anti-Rap (Fzr) antibody generated by us identifies a single band of ~50kd on western blots, consistent with the expected size for the Rap (Fzr) polypeptide (arrow, Figure 24 panel E).

Rap (Fzr) Peptide	Amino Acid sequence
DMF1	SPQTSKKQRDCGETARDS
DMF2	KYQSPTKQDYNGECPY

Table 3: Rap (Fzr) peptides used to generate antibodies

The spatial expression pattern of the Fzr was analyzed by immunohistochemical staining the third instar eye imaginal disc with anti-Rap/Fzr antibody. The Fzr protein is expressed in the posterior part of the eye disc, and in the position of the morphogenetic furrow. Higher magnification revealed a more detailed picture of the Fzr protein expression. In the most posterior region of the eye disc R cell clusters are seen to express Fzr. When the third instar eye imaginal disc was double stained with anti-Fzr (green) and neuron specific anti-elav (red) antibody it showed that the Fzr protein is co-localized with elav in the photoreceptor clusters. Some expression of Fzr protein is also observed in the morphogenetic furrow.

Figure 24. Expression of *fzr* mRNA and protein. In-situ hybridization and antibody staining of *fzr*. Third instar eye imaginal discs were probed with antisense *fzr* (A). The *fzr* mRNA is expressed in the morphogenetic furrow (arrow). Hybridization is also observed in individual clusters of the posterior part of the eye disc (circle in A). Third instar larval eye imaginal disc were stained with anti-Fzr (B). The Fzr protein is expressed in the morphogenetic furrow (arrows in D) as well as in the R cell clusters of the posterior part of the eye disc (circles in C and D). The expression of Fzr antibody overlaps with the expression of elav, a protein that is expressed in the photoreceptor neurons (yellow in D). A western blot (E) of *Drosophila* whole fly extract, shows that the anti-Rap/Fzr antibody recognizes a single polypeptide band with the expected size (~50kd) for the deduced Fzr protein. Varying amounts of the tissue protein was loaded on the SDS-PAGE gel to titrate the antibody activity. Negative controls with pre-immune serum did not yield any specific bands. Positive controls with a neuron specific antibody mAb 8C3 showed the expected band at ~45kd (not shown).



Effects of Targeted misexpression of Rap (Fzr)

Over expression of Rap (Fzr) induces precocious cell cycle exit but neural differentiation is not affected. To examine whether the timing of cell cycle exit is critical for cell growth and pattern formation in the developing eye, I tested the effects of misexpression of *rap (fzr)*. I targeted the expression of *rap/fzr* to cells of the developing eye imaginal disc employing the GAL4-UAS expression system (Brand and Perrimon, 1993). I expressed *UAS-fzr* in the developing eye using an *eyeless-GAL4* driver. The expression of *eyeless (ey)* in the eye-antennal disc is specific to the eye primordial and begins early during the embryonic stages. As a control, I tested the expression of *UAS-lacZ* under the control of the *ey-GAL4* driver (Appendix 4). Premature over-expression of *rap/fzr* resulted in three classes of phenotypes. First, as expected, the development of the compound eye was inhibited due to premature mitotic exit. The extent of the inhibition varied from a drastic reduction of the eye to a complete loss of the eye (Figure 25 C and D).

Induction of ectopic Antenna: An unexpected result of the overexpression of *rap (fzr)* was the induction of ectopic antenna (Figure 25 C and E). Ectopic antennae were induced in the position of the eye and surrounding regions in the head. The ectopic antennae were complete with all the three antennal segments and comparable to the normal wild type antenna (Figure 25 E). In some cases partially formed antennae, with only the first antennal segment were also seen (Figure 25 C).

To test if the induction of the ectopic antenna was due to the activation of the antennal specification genes, I monitored the expression of *Distaless (Dll)*, a

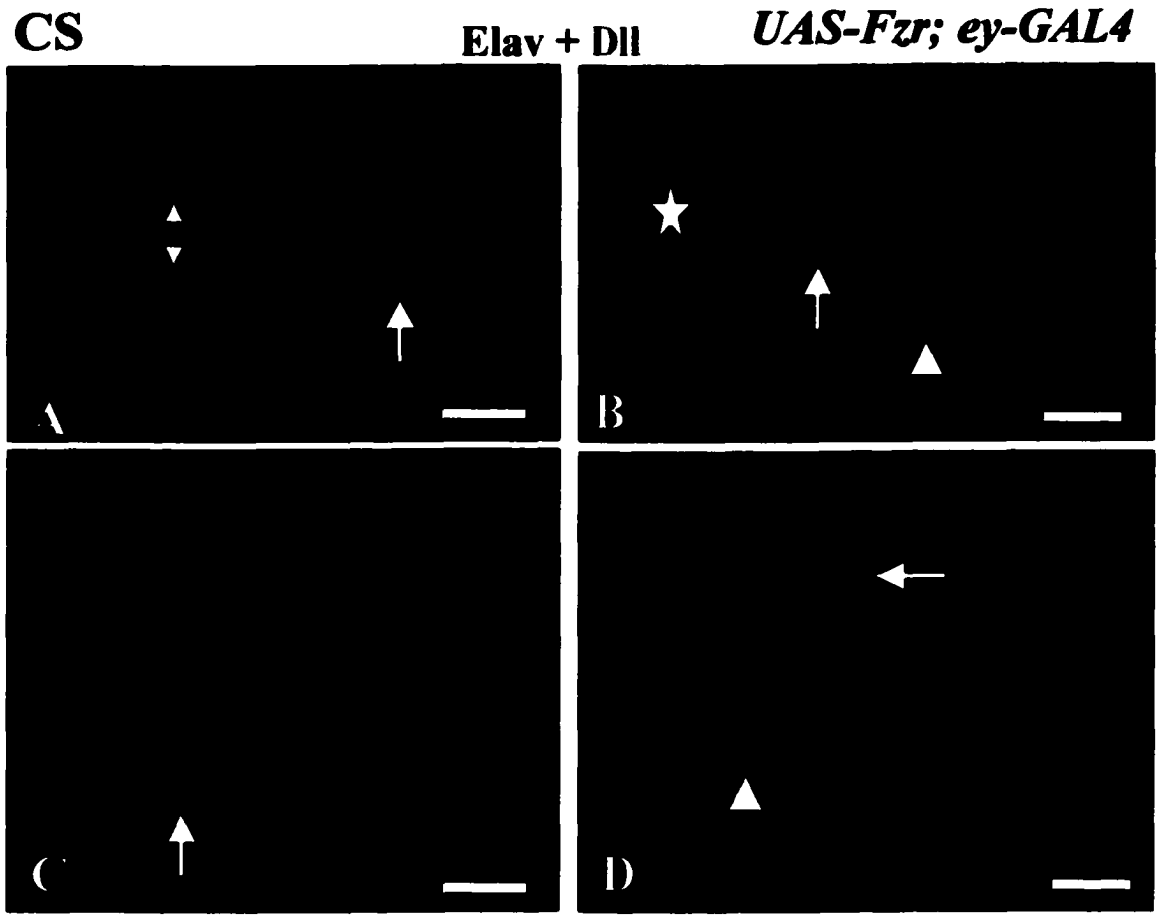
Figure 25. Phenotypic expression of *UAS-fzr; ey-GAL4* construct. When the *UAS-fzr* construct was expressed by the *eyeless GAL4* driver, different phenotypes were observed. SEMs of the compound eyes of *UAS -fzr* (A) and *ey-GAL4* (B), both constructs had a wild type phenotype. The construct *UAS- fzr; ey-GAL4* shows an amalgamated of phenotypes. Some flies did not develop one of their compound eyes (Figure C arrow head). In many other occasions an ectopic antenna develops (red arrows in figures D and E). In other examples the first antenna segment develops an extra structure (arrow in Figure F) and many of these of animals contain an extremely reduced eye (asterisk in Figure F). Other phenotype present in these individuals is the developing of an ectopic compound eye (arrow in Figure G) and the developing of a tumor like structure in the head region (arrow in Figure I). Figures H and J show a higher magnification of these tumors-like structures. (K) anti-Elav and anti-Fzr double antibody staining of the wild type third instar eye imaginal disc. (L) anti-Fzr antibody staining in the *UAS-fzr; ey-GAL4* construct. In the *UAS-fzr; ey-GAL4* construct the expression of Fzr was observed in the antenna disc and it is continuous in the eye disc.

homeotic gene and a key regulator of antennal fate specification in third instar eye-antennal discs (Duncan et al., 1998; Pai et al., 1998). In the wild type eye disc, Dll expression is restricted to the antennal disc (Figure 26 A), and no expression was seen in the eye disc. In third instar eye discs from *ey-GAL4; UAS-fzr* larval, Dll expression is seen in ectopic locations consistent with the induction of ectopic antenna (Figure 26 B). The location and distribution of the ectopic Dll positive cells was random within the eye-antennal disc. The size of the ectopic Dll positive tissue also varied considerably from one eye disc to another. Labeling with phospho-H3 antibodies suggests that the ectopic antennae are derived from the additional proliferation of the antennal precursor cells. These data suggest that premature overexpression of *rap/fzr* leads to an increase in the size of the antennal primordia and which then gives rise to ectopic antennal tissue in the adult.

Targeted over-expression of Rap (Fzr) induces Tumor like structures

Interestingly, overexpression of *rap/fzr* also induced tumor like structures in the position of the eye and more medially on the head of the fly (Figure 25 D and F). To determine the cellular nature of these tumorous growths, I examined sectioned tumors by light microscopy following toludine blue staining. I observed that the induced tumor like structures contained cells larger in size when compared to sections of the wild type tissue in the head region (data not shown). To ascertain whether the increase in cell size was accompanied by increase in DNA content, I stained the third instar eye imaginal discs from *ey-GAL4; UAS-fzr* larval with the DNA-specific dye Hoechst, neuronal marker Elav, and Phalloidin which labels the

Figure 26. Premature Over-expression of *rap /Fzr* induces ectopic expression of the antennae specification protein Distaless in the developing eye-imaginal disc: Phenotype in the developing eye imaginal disc. Third instar eye discs from wild type (A), and *ey-GAL4*; *UAS-fzr* (B), were stained with anti-Elav (red) and anti-Dll (green). In the wild type (A), Elav expression (red) is specific to the eye disc and highlights the ordered pattern of the developing R cell clusters posterior to the MF (double arrow head). The antennal precursors are highlighted by the specific Dll expression (green) in the antennal disc. (B), In the *ey-GAL4*; *UAS-fzr* a very small number of Elav positive R cells (red) are seen. The R cell clusters are abnormally large. Dll staining in the antennal disc shows a large field of ectopic Dll positive cells (arrow) in addition to the endogenous antennal precursors (arrow head). C, D. Premature over expression of Rap (Fzr) does not inhibit cell growth and cell differentiation. Third instar eye imaginal disc were stained with the eye differentiation marker Elav (green), and the actin marker Phalloidin (red) to out line cells. To visualize the DNA content, cells were label with the Hoechst dye (blue). In the wild type, C, distinct domains of Elav (green) and Actin (red) staining are seen. DNA labeling (blue) is uniform through the disc. In (D) the ey-GAL4; UAS-fzr disc, abnormally large neuronal cell clusters express Elav (red) and also stain with Hoechst (blue). The DNA staining shows abnormally large nuclei presumably due to endoreplication. Anterior to the right. Bar=10μ.



Elav + Phalloidin + Hoechst

Actin cytoskeleton of the cells. Confocal images show that the Elav positive neurons in the eye disc are abnormally large and also show strong staining with Hoechst (Figures 26 C and D). Expression patterns of the pro-neural antigen Senseless (Figure 28), neuronal antigen Elav show that overexpression of *rap/fzr* does not inhibit neural differentiation (Figure 28 B, green). The frequency of induction of ectopic antennae was about 75-80% (n=200 eye-antenna disc scored) when examined in the third instar larval and 30% in the adult flies (n=1074) (table 4). The *ey-GAL4*; *UAS-fzr* progeny exhibited 50-60% pupal lethality.

Ectopic expression effects with other of GAL4 lines

I have tested large number other GAL4 enhancer lines for the effects of the ectopic expression of Rap (Fzr) [see table]. Among these, expression by some of the GAL4 lines was completely lethal at the larval and pupal stages. Expression of *rap (fzr)* with the *dachsund (dac)-GAL4* had phenotypic effects similar to the *ey–GAL4*. However, expression by the *dac-GAL4* resulted in a higher incidence of pupal lethality. The other *GAL4* lines tested did not have significant effects on the patterning in the eye (Figure 27).

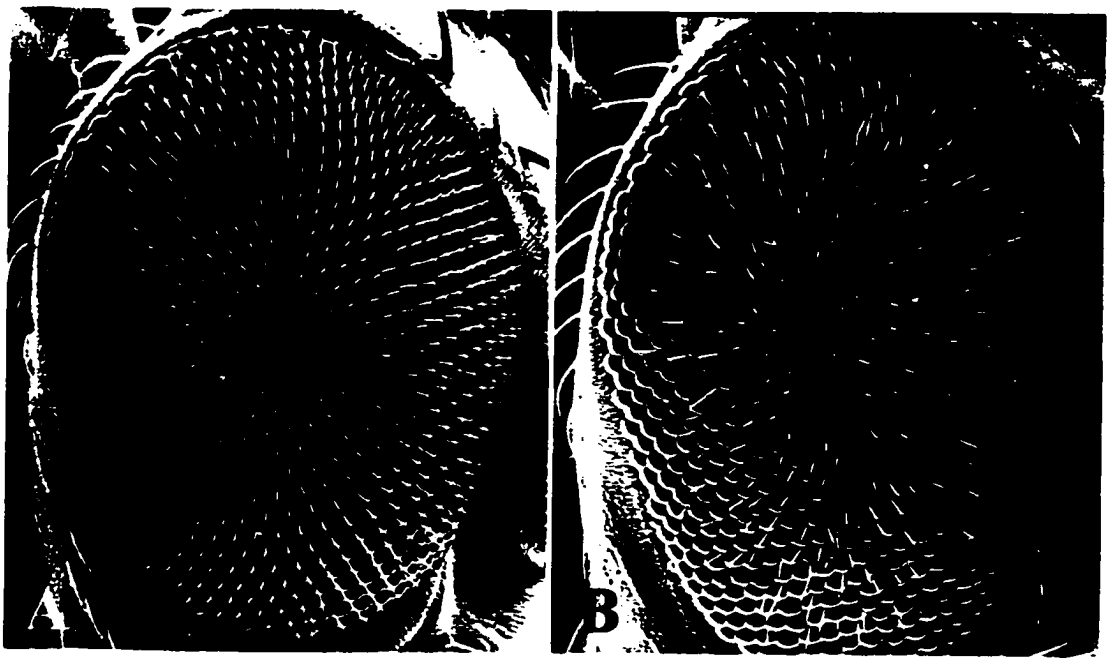
Neural differentiation is not inhibited in the *UAS-rap/fzr; ey-GAL4* construct

To study if the precocious mitotic exit affects neural differentiation in the *UAS-rap/fzr; ey-GAL4* I double stained the third instar eye imaginal disc from the *UAS-rap/fzr; ey-GAL4* with an antibody against the proneural protein Senseless and against the neural protein Elav (Nolo et al., 2000). The 'senseless' protein is a nuclear protein with four Zn fingers. The expression of this protein is required in the

sensory organ precursors (SOP) for proper proneural gene expression (Nolo et al., 2000). The expression pattern of Senseless and Elav shows that the premature cell cycle exit, induced by the misexpression of *UAS-rap/fzr* with the *ey-GAL4* domain, does not inhibit neural differentiation.

When the *UAS-rap/fzr* was expressed using the *elav* (*embryonic lethal abnormal visual system) GAL4 (elav-GAL4)* the F_1 progeny had a rough eye phenotype (Figure 27). *elav* encodes a RNA binding protein that is expressed right after the neuronal birth and is required for the development and maintenance of the nervous system (Koushika et al., 1996; Yao and White, 1994).

Figure 27. Expression of *UAS-fzr* driven by *elav-GAL4* driver results in a rough eye phenotype. A) *UAS-fzr*, B) *UAS-fzr; elav-GAL4* .



Rough Eyes	Missing Anthenas	Reduced Eyes rough and small	Extrenmly reduced eyes	No eye No Double Anthena	Extremely reduced eye with double or Ectopic anthena	Extremely reduced eye with tumor	Tumor	Double anthena
Totals of Females								
224	0	112	18	1	36	34	117	74
Totals of Males								
114	8	135	22	2	34	29	88	26
Total 338	8	247	40	3	70	63	205	100

Table 4. **Summary of the Effects of Targeted missexpression using the Gal4-UAS targeting system**. The UAS-*Rap*/Fzr construct was crossed with different Gal4-contructs. Twenty two Gal4 were used in this experiment. UAS-*rap*/fzr only showed a missexpression phenotype with 4 of these Gal4; *elav*, *hairy*, *dachshund* and *eyeless*.

Total Number of individuals: 1,074.

Figure 28. Neural differentiation is not affected by premature cell cycle exit. Anti-senseless antibody was used to follow the pattern of expression of the proneural, Senseless protein and the neural antigen Elav. In the wild type (*CS*) the expression of senseless (green) occurs as a circular pattern in the antennal disc. Senseless expression precedes the expression of the elav protein (red). In the *UAS-fzr; ey-GAL4* discs the expression of Senseless and Elav suggests that neural differentiation is unaffected.

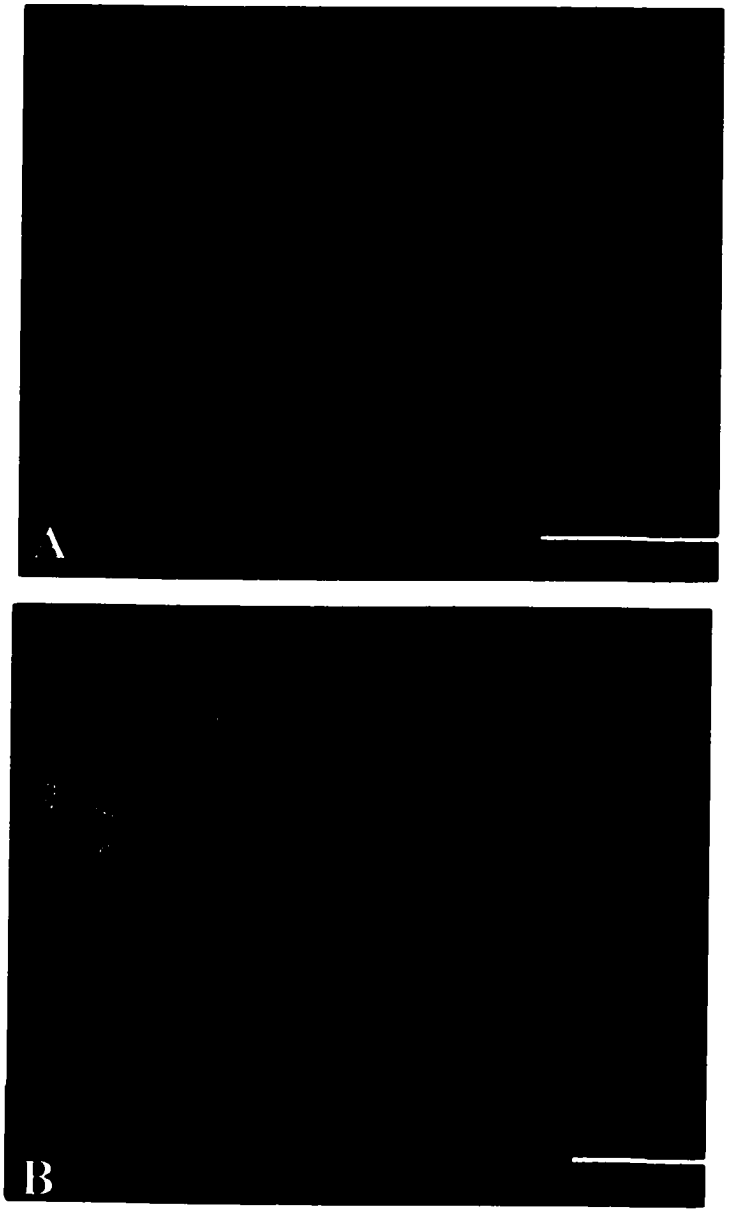
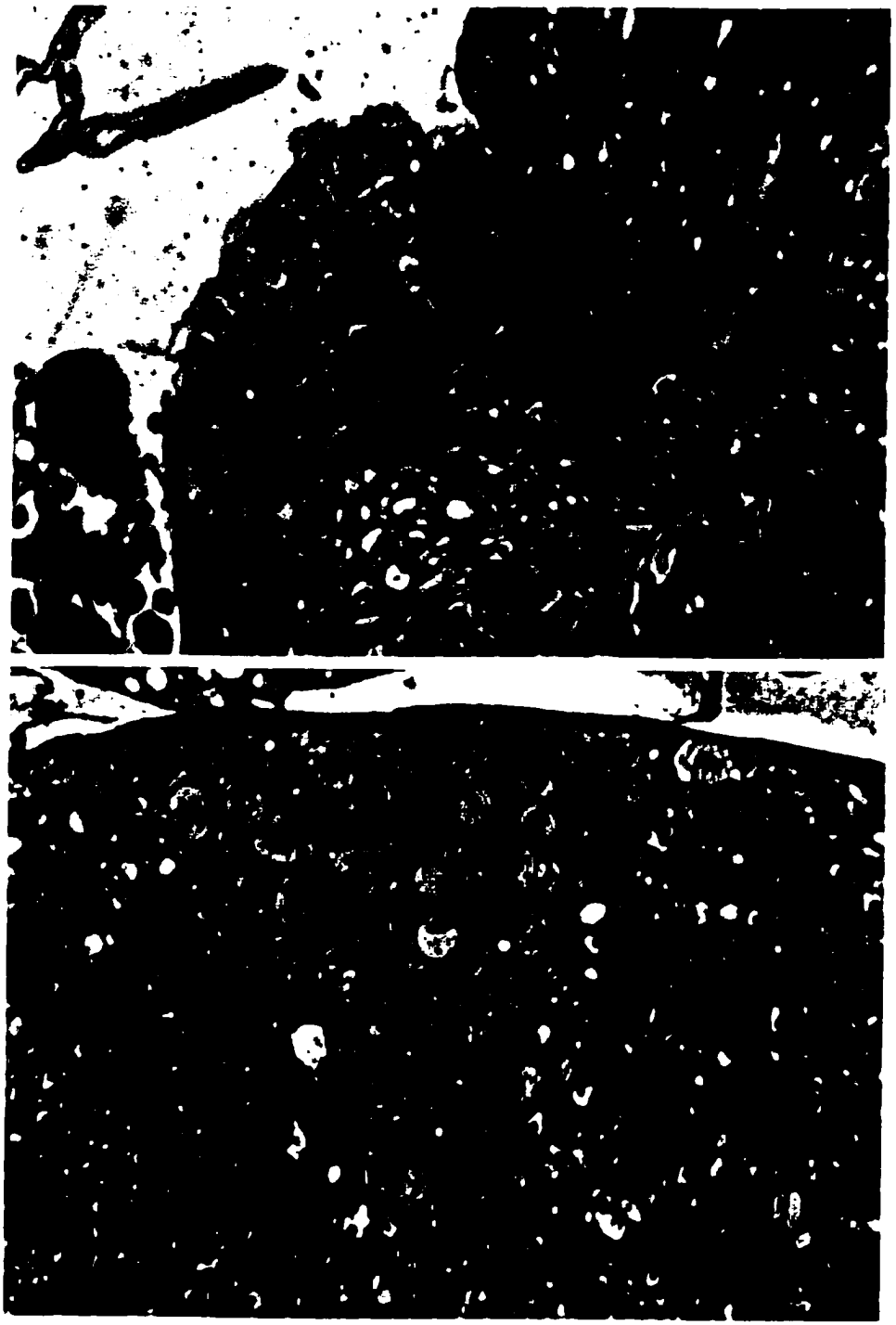


Figure 29. Anatomy of the ectopic tumors induced by misexpression of Rap/Fzr. A) A plastic section of the wild type head region. B) A plastic section of the tumor tissue showing large nuclei when compared to wild type tissue from a similar region of the head (B).



Discussion

Correct pattern formation requires, in addition to proper cell fate specification, precise coordination of cell cycle events with processes regulating growth and cell number. Although these events are tightly coupled, classic experiments in yeast (Hartwell, 1971) have showed that these events are separable, and further, that growth control is dominant over the machinery that regulates cell cycle and is rate limiting. Recent experiments in the *Drosophila* wing support these conclusions and have shown that cell cycle arrest leads to continued growth and cell death (Neufeld et al., 1998; Weigmann et al., 1997). To examine whether the precise timing of cell cycle exit is critical for correct pattern formation in the fly eye I have examined the role of *rap/fzr* during eye development. My results show that loss-of-function mutations in *rap/fzr* result in mis-regulation of cell cycle events and that the cells, which fail to exit the cell cycle, go through additional abnormal mitotic cycles.

Premature overexpression of *rap (fzr)* led to precocious cell cycle exit by the retinal precursors but it did not affect cell growth and differentiation events. In *ey-GAL4; UAS-fzr* eye imaginal discs, a small number of abnormally large photoreceptor neurons were typically formed, consistent with my interpretation that DNA endoreplication and cell growth continue unaffected in cells that exit mitosis precociously. This finding is similar to the results reported for premature *fzr* overexpression in the developing *Drosophila* wing disc where entry into mitosis was inhibited prematurely and resulted in DNA endoreplication and abnormally large cells (Sigrist and Lehner, 1997). In the wing

imaginal disc, neither premature mitotic exit nor cell cycle arrest had any effect on pattern formation (Neufeld et al., 1998; Weigmann et al., 1997). My results with the loss-of function mutants in the developing eye disc are also in agreement with the results reported in the developing embryo (Sigrist and Lehner, 1997). However, my results with the developing eye differ significantly in two important aspects. First, premature mitotic exit results in the induction of ectopic antennae, and second, cells that exit mitosis prematurely endoreplicate and differentiate into abnormally large cells which form tumor like structures. Recent studies have shown that defects in cell cycle checkpoints and mutations in signaling pathways regulating cell proliferation lead to changes in cell size and contribute to tumorigenesis without affecting cell differentiation (Potter et al., 2001; Tapon et al., 2001).

The eye antennal disc is unique among imaginal discs because it gives rise to more than one adult organ. In addition to the eye and the antenna, other cephalic structures of the adult fly are also derived from the eye antenna imaginal disc. The anlagen for the eye-antennal disc arise as a group of about twenty cells in the embryo and proliferate through the larval stages. Cell lineage and mosaic analyses studies (Morata and Lawrence, 1978; Morata and Lawrence, 1979; Postlethwait and Schneiderman, 1971) have suggested that a clonal restriction between eye and the antenna arises early during the embryonic stages. Recent studies have shown that the specification of the eye and antenna does not take place until the second larval instar (Kumar and Moses, 2001). An intriguing question is how the relative sizes of the presumptive eye and the antennal developmental fields are regulated during morphogenesis. My results here suggest that timely cell cycle exit is a critical factor.

When the presumptive eye field is reduced or restricted in size by premature cell cycle exit induced by the expression of *rap/fzr*, the size of the presumptive antennal field apparently increases, resulting in the induction of ectopic antenna. The converse experiment (wherein the antennal precursors are induced to exit cell cycle) precociously is not feasible at the present time due to the lack of appropriate GAL4 drivers. These data suggest that cell cycle regulatory mechanisms, which are mutually inhibitory to eye-antenna fields, may govern the respective sizes of the individual developmental fields, which then dictates pattern formation in the developing tissue. Such regulatory mechanisms could be mediated by the Notch and EGFR pathways, for example, which have been shown to play an important role in cell proliferation and pattern formation and act antagonistically in the specification of the eye and the antenna (Kumar and Moses, 2001; Kurata et al., 2000).

Materials and Methods

***In-situ* hybridization.**

10µl of the 1µg *fzr* cDNA (appendix 1) was added to 2.5µl of ddH_2O, 1.5 of restriction buffer of *Eco RI*, and 1.0µl of *EcoRI* restriction enzyme. Another digestion was done as; 10µl of the *fzr* cDNA (1µg) was added to 2.5µl of ddH_2O, 1.5µl of restriction buffer of *Hind III*, and 1.0µl of *Hind III* restriction enzyme. The reactions were done at 37°C for 2 hours. Afterwards the samples were incubated for 30 minutes at 65°C to inactivate the restriction enzymes. The samples were extracted with 1:1 phenol/chloroform, followed by one extraction with chloroform. To the aqueous phase equal amount of 4M NH4OAc were added followed by three volumes of absolute ethanol then the samples were incubated at –70°C for one hour. The precipitated DNA was recovered by centrifugation at 10,000g in a Sorvall centrifuge. The supernatant was discarded and the DNA was washed twice with 70% ethanol. The DNA was dried using a Speed Vac for 5 minutes and resuspended in 13µl of diethyl pyrocarbonate treated ddH_2O. The *fzr* cDNA was labeled as described in the DIG RNA Labeling Kit (Roche Catalog # 1175025). The labeling reaction was done as described below; 2µl of 10X transcription buffer, 2µl NTP labeling mixture, 2µl of Sp6 RNA polymerase, 1µl of RNase inhibitor solution were added to the 13µl of the *EcoRI* digested *fzr* cDNA, (the final volume was 20µl). A second labeling reaction was done as; 2µl of 10X transcription buffer, 2µl NTP labeling mixture, 2µl of T7 RNA polymerase, 1µl of RNase inhibitor solution were added to the

13µl of the *Hind III* digested *fzr* cDNA, (the final volume was 20µl). Both reactions were incubated at 37°C overnight. The next day 2µl of DNase I (10units/µl and RNase free) were added to the reaction and left incubated for 15 minutes at 37°C. 2.5µl of 4M LiCl were added followed by 75µl of absolute ethanol. Then, both tubes were incubated at –70°C for 1 hour. The tubes were centrifuged at 12,000g in a Sorvall centrifuge for 15 minutes at 4°C. The supernatant was discarded and the RNA was washed twice in 70% ethanol. The RNA was dried using a Speed Vac. The labeled RNA was resuspended in 100µl of diethyl pyrocarbonate treated ddH_2O followed by incubation at 37°C for half hour. The samples were kept at –20°C. Third instar larva were dissected in Ringer's solution: (46 mM NaCl, 182 mM KCl, 10 mM Tris-HCl (pH 7.2), $NaHCO_3$, 3.0 mM $CaCl_2.2H_2O$, 1.0g glucose). The brain and mouth hook were left attached to the eye disc to help to protect them. These brain/eye disc complexes were fixed for 15 minutes in 4% formaldehyde in 1X PBS at room temperature. This was followed by a second fixation in 4% formaldehyde in 1X PBS: 0.6% triton X100 for 15 minutes at room temperature. The tissue was washed three times in 1X PBS: 0.1% tween-20 for 5 minutes at room temperature. The tissue was digested with 1:2500 dilution of 12.5 mg/ml of proteinase K in 1X PBS: 0.1% tween-20 for 5 minutes at room temperature. To stop the digestion the tissue was immersed in 20 mg/ml of glycine in 1X PBS: 0.1% tween-20 for 10 minutes. This was followed by two washes in 1X PBS: 0.1% tween-20. The tissue was post fixed for 15 minutes at room temperature in 4% formaldehyde; 0.1% glutaraldehyde in 1X PBS. The tissue was washed 5 times for 5 minutes each at room temperature in 1X PBS; 0.1% tween-20. Thereafter the complexes were washed for 10 minutes in 1:1 1X PBS; 0.1% tween-20: Hybridization buffer (50% formamide, 5X SSC, 0.1 µg/ml salmon sperm

DNA, 0.1 μg/ml tRNA, 0.1% Tween-20, 0.05μg/ml heparin). The tissue was transferred to a single well (of a 96 well cell culture dish) and pre-hybridized in 50 μl of hybridization buffer at 50°C for 1 hour. 50ng of two probes, sense and antisense strands, were denatured in separated tubes in 50μl of hybridization buffer (by boiling them for 10 minutes) and added to different groups of tissues. The tissue was then incubated for twenty hours at 50°C. Thereafter the tissue was washed three times for 20 minutes in hybridization wash buffer (50% formamide, 5X SSC, 0.1% Tween-20 in H_2O) at 50°C each. The tissue was then incubated in 3:1, 1:1, 1:3 hybridization wash buffer: 1XPBS; 0.1%Tween-20, for 20 minutes each at 50°C. The tissue was washed 4 times for 20 minutes each in: 1XPBS; 0.1%Tween-20 at 50°C. The tissue was then incubated with 1:2000 dilution of alkaline phosphatase conjugated anti-digoxigenin antibody (Roche Catalog # 1093274). This incubation was done overnight at 4°C. The tissue was then washed 4 times in 1XPBS; 0.1%Tween-20 at room temperature for 20 minutes each. This was followed by incubation in staining solution (0.1M Tris-HCl, 0.1M NaCl, 0.05M $MgCl_2$) for 5 minutes at room temperature. I performed the color reaction by immersing the tissue in the color solution; 200 μl of NBT/BCIP solution (Roche, Catalog # 1681451) in 9.8 ml of staining solution. This incubation was done for 24 hours at room temperature. The color reaction was stopped by incubating the tissue in 1XPBS at room temperature.

Inverse PCR.

The inverse PCR was performed as described in the Berkley *Drosophila* Genome Project with some modifications (Ochman et al., 1998). First a quick fly genomic DNA

preparation was done as follows. Thirty anesthetized flies of the P2241 lethal were collected in an eppendorf tube and frozen at -80°C for one hour. The flies were ground in 200µl of buffer A (100mM Tris-HCl, pH 7.5, 100mM EDTA, 100mM NaCl, 0.5% SDS) with a disposable tissue ground. 200µl more of Buffer A were added and the tissue was ground until only cuticles remained. The homogenate was incubated at 65°C for 30 minutes. This was followed by the addition of 800µl of Li/KAc solution (1 part 5M KAc stock: 2.5 part 6M LiCl) and incubated in ice for 1 hour. The homogenate was spun for 15 minutes at room temperature. One ml. of the supernatant was transferred to a new eppendorf tube avoiding floating crude. 600µl of isopropanol were added and mixed. The tubes were spun for 15 minutes at room temperature. The supernatant was removed and the white precipitate was washed twice with 70% ethanol. The pellet was dried in the Speed Vac for 5 minutes. The pellet was resuspended in 150µl TE buffer, pH 7.4 (10mM Tris-Cl pH 7.4, 1mM EDTA pH 8.0). The DNA was stored at -20°C. From this DNA preparation two separate digestions were prepared. The first digestion was consisted of 10µl of P2241 genomic DNA (~ 2 flies), 2.5µl of *MspI* restriction buffer, 2.0µl of 100µg/ml RNase (heat treated), 0.5µl (10 units) *MspI* enzyme and 10µl of ddH_2O. The second digestion was done as; 10µl of P2241 genomic DNA (~2 flies), 2.5µl *Sau3A* restriction buffer, 2.0µl of 100µg/ml of RNase (heat treated), 2.5µl of 10X BSA, 2.5µl of *Sau3A* restriction enzyme (~10 units) and 5.5µl of ddH_2O. Both reactions were incubated for 2.5 hours at 37°C. Thereafter they were incubated at 65°C for 20 minutes. Two separate ligation reactions were done, (one for each digestion) using the commercially available DNA Ligation Kit from Stratagene (catalog # 203003). For the first ligation 10µl of the *MspI* digested P2241 genomic DNA (~1 fly) was added to 40µl

of 10X ligation buffer, 40µl of 10mM rATP, 311µl of ddH_2O and 1µl of ligase (2 Weiss units). For the second ligation 10µl of the *Sau3A* digested P2241 genomic DNA (~1 fly) was added to 40µl of 10X ligation buffer, 40µl of 10mM rATP, 311µl of ddH_2O and 1µl of ligase (2 Weiss units). Both reactions were incubated overnight (~16 hours) at 4°C. Monomeric circles of ligated DNA were created (Collins and Weissman, 1984) many of them containing the P element construct with regions of the DNA sequences flanking element. The ligated DNA was ethanol precipitated by adding equal amount of 4M NH_4OAc (400µl), followed by twice the amount of absolute ethanol (800µl). The samples were incubated at –70°C for 1½ hours. The samples were centrifuged at 10,000 g in a Sorvall centrifuge for 15 minutes at 4°C, the supernatant was discarded and the white precipitate was washed twice with 70% ethanol. The DNA was dried using the Speed Vac for 5 minutes and resuspended in 150µl of TE buffer (~1fly per 150µl). Two PCR reactions were set up for each enzyme digestion. For each of the enzyme digestions I set up two PCR reactions. The first one consisted of 10µl of the restriction digested P2241 genomic DNA (*MspI* or *Sau3A*), 1.5µl of 10mM dNTP mixture, 5µl of 10X buffer, 1µl of 50mM Magnesium Sulfate, 24µl ddH_2O, 1µl of 25mM MgSO4, 4µl of 10µM *Plac1* primer (**CAC CCA AGG CTC TGC TCC CAC AAT**), 4µl of 10µM *Plac4* primer (**CAA TCA TAT CGC TGT CTC ACT CA**), and 0.5µl (1.25U) of the Platinum® *Pfx* DNA Polymerase (Life Technologies Catalog # 11708013). The second consisted of 10µl of the restricted digested P2241 genomic DNA (*MSPI* or *Sau3A*), 1.5µl of 10mM dNTP mixture (Gibco BRL Catalog # 18427-013), 5µl of 10X Buffer, 1µl of 50mM Magnesium Sulfate, 24µl ddH_2O, 1µl of 25mM $MgSO_4$, 4µl of 10µM *Pry4* primer (**CAA TCA TAT CGC TGT CTC ACT CA**), 4µl of 10µM *Plw3-1* primer (**TGT CGG**

CGT CAT CAA CTC C), and 0.5µl (1.25U) of the Platinum® *Pfx* DNA Polymerase (Life Technologies Catalog # 11708013). The PCR reaction was done in a MJ Research Programmable Thermal Controller, model PTC 100. The parameters used were; 1X 95°C for 5 minutes, 35X 95°C for 30 seconds each followed by 1 minute at 60°C (annealing temperature for primers combination *Plac4/Plac1*), 1X 72°C for 10 minutes, 4°C hold. The second parameter used was; 1X 95°C for 5 minutes, 35X 95°C for 30 seconds each followed by 1 minute at 55°C (annealing temperature for primers combination *Pry4/Plw3*-1), 1X 72°C for 10 minutes, 4°C hold. 5µl of the reaction was analyzed in 0.7% agarose gel in 0.5X TBE buffer (0.045M Tris-borate, 0.001M EDTA). The concentration of the PCR product was analyzed by comparing the intensity of the PCR product band with the intensity of the various bands of the Stratagene DNA Kb ladder. For sequencing, the inverse PCR products were purified using the QIAquick PCR Purification Kit (Qiagen catalog # 28104). To analyze the quality and quantity of the purified inversed PCR product, 5µl of each sample were loaded in a 0.7% agarose gel. PCR product of the samples were prepared for sequencing as follow: 30ng of the PCR product (for products with size of 200-500bp) or 50ng of the PCR product (for product with size of 500-1000) 4 picomoles of the *Splac2/Sp1* primers; (*Splac2*: **GAA TTC ACT GGC CGT CGT TTT ACA A**; Sp1: **ACA CAA CCT TTC CTC TCA ACA A**) were added to the PCR product obtained with the *Plac4/Plac1* primer. *Spep1* and *Sp6* (*Spep1*: **GAC ACT CAG AAT ACT ATT C**, *Sp6*: **TGA CCA CAT CCA AAC ATC CTC TT**) were added to the PCR product obtained with the *Pry4/Plw3-1* primers. The final volume of each sample was 12µl. The samples were sequence using the BigDye Terminator Cycle Sequencing. The samples were cycled in Gene Amps PCR System

9600s (or 9700s). The reactions were electrophoresed on ABI Prism 377XL (or 3700) DNA Sequencer at Rockefeller University. The sequences obtained were compared with the *Drosophila* Genome DNA database at FlyBase were they were blasted.

Southern Blot Hybridization

The genomic DNA was isolated using the rapid small-scale DNA isolation method of Herman Steller with some modifications. 50 flies of each allele were collected in an eppendorf tube. 500µl of solution A 0.1M Tris-HCl, pH 9.0; 0.1M EDTA; 1%SDS and 0.5-1% Diethyl Pyrocarbonate were added and the flies were homogenized. The homogenate was incubated at 70°C for 30 minutes in a water bath. 70µl of 8M Potassium Acetate were added to each tube and they were incubated on ice for 30 minutes. The samples were centrifuged at 4°C at 10,000g in the Sorvall centrifuge for 15 minutes. The supernatant was transferred to a clean tube. A Phenol/Chloroform (1:1) extraction was performed twice followed by a chloroform extraction. 0.5 volume of isopropanol at room temperature was added, mixed and immediately centrifuged for 5 minutes at room temperature. The supernatant was discarded and the precipitate was washed twice with 70% ethanol. After discarding the last 70% ethanol wash, the DNA was dried. The Genomic DNA was resuspended in 100µl of TE buffer. The restriction digestion was done in 20 µl reaction volume with 500 µg of genomic DNA from each allele were added to 2µl of 10X restriction buffer, 1µl of the restriction enzyme and ddH_2O to complete a 20µl volume. The reaction was carried out at 37°C for 2 hours. After the digestion 4µl of 6X type III loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF and 30% glycerol in H_2O) were added to each sample and loaded in a 0.7% agarose gel prepared in

0.5X TBE (0.045M Tris-borate, 0.001M EDTA). Stratagene DNA Kb ladder (catalog # 201115) were loaded as a molecular weight reference. The gel was run overnight at 22 volts. The next day the gel was photographed and then transferred to a Hybond™ N+; positively charged nylon filter (Amersham Life Sciences) using alkaline capillary transfer with 0.4M NaOH as the transfer buffer. The transfer was done overnight. Then, the membrane was incubated in 0.4M NaOH for 20 minutes to bind the DNA to the membrane. Finally the filter was washed briefly in 5X SSPE buffer. The *fzr* probe was radioactively labeled using the Random Prime labeling Kit (Roche Biochemical Corp. Catalog # 1004760) using the following procedure: 1µl of denatured *fzr* $cDNA_1$ (2.6kb) was added to 1µl of dATP, 1µl of dGTP, 1µl of dTTP, 5µl of ^{32}P dCTP (50µCi), 2µl of reaction buffer, 1µl of Klenow enzyme, and autoclaved ddH2O to complete a 20µl volume. The labeled reaction was incubated for two hour at 37°C. Two µl of 0.2M Potassium Acetate were added. The efficiency of the labeling was determined by TCA precipitation. 1µl of the labeled DNA was taken and diluted in 500µl of ddH_2O. Subsequently 10µl of this dilution were taken immediately and added 500µl of to 10mg/ml of Salmon Sperm DNA. This solution was filtered through a 47mm Glass Microfibre Filter (Whatman Catalog # 1820047). The filter was dried and transferred into 15ml of universal cocktail. The sample was quantified in a Scintillation counter, and the efficiency and the activity of the probe were calculated. The Filter was pre-hybridized in the following solution; deionized 50% Formamide, 5X SSPE, and 5X Denhardt's solution; 0.5% of SDS, 100µg of denatured Salmon Sperm DNA, in a rotator incubation at 42°C. This solution was filtered through a 0.45µ cellulose acetate filter and pre-warm at 37°C. The filter was pre-hybridized for one hour, time after the denature

probe was added. The hybridization was done overnight at 42°C. The filter was washed in 2X SSPE; 0.5% SDS immediately followed by another wash of 2X SSPE; 0.1% SDS both of this washes were done at room temperature. The third wash was done by incubating the filter in 0.1X SSPE; 0.5 SDS for one hour at 37°C in a water bath shaker. The fourth wash consisted of 0.1X SSPE; 0.5% SDS for 3-4 hours at 68°C in a water bath shaker. The last wash was done by briefly incubating the filter in 0.1X SSPE at room temperature. The filter was covered in Saran Wrap, and exposed using a Kodak Biomax MR film (catalog # 8952855).

EMS Mutagenesis

300 adult Cs (Cantonese special) males (wild type) were collected. These animals were starved overnight in empty bottles at 23°C. In the fume hood 3mm filter papers were put in the bottom of clean bottle and soaked in a solution containing 25mM Ethane Methyl Sulfonate (EMS), 10mM Tris pH 7.5 and, 1% sucrose. The starving (and dehydrated) males were transferred to the bottles with the EMS solution. These flies were left feeding overnight. The next day the males were transferred to empty bottle to allow them to clean themselves off the chemicals. After 30 minutes they were transferred to bottles with regular cornmeal food. They were allowed to recover for about 6 hours. The males were separated in groups of twenty and crossed to double the number of *y,Sc,rap,v,f,* homozygote females. The crosses were transferred to fresh bottles of food every day for 5 consecutive days after which they were discarded. The females from the F1 were screened for red and rough eye phenotype. The rough eye flies were crossed to FM7 females to establish a stock. The progeny of these crosses were observed to verify that they kept the rough phenotype (only germ line mutants will keep the phenotype). Males

from these crosses were crossed again to various alleles of *rap* to verify their lack of complementation for the *rap* locus. Also they were crossed to wild type flies (Cs) to rule out dominant mutations.

Plasmid rescue.

50 flies of the P2241 lethal stock were used to extract DNA using Herman Steller procedure as described above. 10μl (5 flies) of the Genomic DNA were digested with EcoRI and Xba1 enzyme at 37°C for 2 hours. The Enzyme was heat inactivated by incubating the tubes at 65°C for 20 minutes. The DNA was ethanol precipitated as described above. The digested DNA was resuspended in 50μl of TE pH 7.4. 25μl of this DNA were used in the ligation reaction. This DNA was ligated using the Stratagene ligation Kit (catalog # 203003). The ligation was done as follows; 25μl of the digested P2241 genomic DNA (~5 flies) were added to 20μl of 10X ligation buffer, 20μl of 10mM rATP, 134μl of ddH_2O and 1μl of ligase (2 Weiss units). The reaction was incubated overnight (~16 hours) at 4°C. 20μl of 3M sodium acetate were added and the DNA was precipitated with 2 volumes of absolute ethanol. The DNA was spun at 10,000g, washed twice with 70% ethanol and dried using the Speed Vac. The ligated DNA was used to transform Epicurian Coli® XL1-Blue MRF' Electroporation-Competent cells (Stratagene cat. no. #200158). 1μl of the P2241 ligated DNA was added to 40μl of the Electroporation competent cells and mixed. It was left on ice for about one minute. This mixture was transferred to a chilled 0.1 cm Electroporation cuvette and electroporated in a Bio-Rad Gene Pulser® at 1700 volts for three seconds. The cuvette was removed from the chamber and 960μl of sterile SOC medium (pre-warmed at 37°C) was added immediately. This cell suspension was transferred to a 15 ml Falcon polypropylene tube

(catalog # 2059) and incubated at 37°C in constant shaking (225 rpm) for one hour. Using a sterile spreader 10 µl of the cells were spread on LB-Amp (50 µg/ml) plates. These plates were incubated overnight at 37°C. Single colonies were selected and inoculated on 10ml of liquid LB medium with ampicillin (50µg/ml) and incubated overnight at 37°C. The culture was pelleted in 1.5ml eppendorf tube at 4°C for 3 minutes (10,000 RPM). The pellet was resuspended in 110 µl of STETL buffer. The tube was incubated in a boiling water bath for 30 seconds, and immediately spun in a microfuge at 4°C for 15 minutes (10,000 RPM). The pellet was removed and discarded with a sterile toothpick. The supernatant was RNase treated by adding 1µl of 5mg/ml RNase A (heat treated). 110µl of isopropanol were added to the supernatant followed by immediate centrifugation for 15 minutes at room temperature (10,000 RPM). The pellet was resuspended in 2.5µl of TE buffer. The DNA was extracted twice with phenol-chloroform (1:1) and once with chloroform. Equal volume of 7.5M ammonium acetate was added followed by 2.5 volumes of absolute ethanol. The DNA was incubated on ice for one hour and spun at 4°C for 20 minutes. The DNA was washed twice with 1ml of 80% ethanol followed by 1 minute spin. The pellet was dried using a Speed Vac, and resuspended in 15 µl of 10mM Tris (pH 7.5) and 0.1mM EDTA. The DNA was restriction digested and analyzed on a 0.7% agarose gel. The Plasmid was used as a template and PCR was done as described above using the same combination of primers as in the inversed PCR procedure. Five µl of the PCR product was analyzed on a 0.7% agarose gel and the rest were purified as described above and submitted for sequencing. The sequences obtained, were analyzed by blasting them to the *Drosophila* genome database.

5-Bromo-2-DeoxyUridine Staining: Third instar larval eye discs were dissected in *Drosophila* Schneider's medium (Gibco cat # 11720-034). The tissue was incubated in Schneider with 75 μg/ml 5-bromo-2'-deoxy-uridine, (BrdU, Roche Catalog No. S 7-13) diluted from a stock of 7.5 mg/ml made fresh in 80% ethanol, for 2 hours at room temperature. Thereafter, the tissue was washed for 5 minutes in Schneider's medium followed by two washes in 1X PBS for 5 minutes each at room temperature. The tissue was then fixed for 15 minutes in 4% formaldehyde in 1X PBS, this was followed by 15 minutes in 4% formaldehyde in 1XPBS; 0.6% Triton X-100 at room temperature. The eye discs were washed 2 times for 15 minutes in 1X PBS; 0.6% Triton X-100 at room temperature. They were washed 2 times for 5 minutes in DNase I buffer: 66mM Tris pH 7.5, 5mM $MgCl_2$, 1mM 2-mercaptoethanol. The discs were incubated for 1 hour at 37°C in 100 units of RNase free Deoxyribonuclease I (DNase I), enzyme (Roche catalog # 776785) in 0.5 ml of DNase I buffer. The tissue was washed 2 times for 10 minutes in 1X PBS; 0.3% Triton X-100 at room temperature. Thereafter, the tissue was incubated in monoclonal anti-BrdU 1:1000 (Sigma catalog # B2531), and rabbit generated anti-phosphate H3 antibody diluted at 1:1000 (Get cat # Company) both in 1X PBS; 0.3% Triton X-100 overnight at 4°C. Then the tissue was washed 2 times for 5 minutes in 1X PBS; 0.3% Triton X-100 at room temperature. The tissue was then incubated in the secondary antibodies at1:200 dilution in 1X PBS; 0.3% Triton X-100 for three hours at room temperature. Finally the eye discs were washed two times for 15 minutes in 1X PBS 0.3% Triton X-100 at room temperature. The discs were mounted in 0.4% of n-

propyll gallate in 1X PBS and Glycerol (0.0825g of n-propyl gallate; 15ml of 1XPBS and 5ml of Glycerol).

Immunohistochemistry of the third instar larval eye imaginal disc and antenna disc: Third instar larval were dissected in 1X PBS. The tissue was fixed in 4% paraformaldehyde in 1XPBS pH 7.4 for 15 minutes at room temperature. Afterward, they were washed in 1X PBS three times at room temperature for 5 minutes each. This was followed by incubation in 1X PBS; 0.6% Triton X100 for 10 minutes at room temperature. For the triple staining, the eye discs were incubated with 1:2000 mouse generated anti β-galactosidase, 1:2000 dilution of rabbit generated anti CycB (generously provided by Dr Christian Lehener, University of Bayreuth) and 1:200 of rat generated anti-elav. All these antibodies were diluted in 1X PBS; 0.3% Triton X100. These incubations were done at 4°C overnight. After the incubation with the primary antibodies the tissue was washed three times with 1X PBS; 0.3% Triton X100, at room temperature for 5 minutes each. The tissue was incubated in the secondary antibodies as follow; 1:200 dilution of anti rabbit FITC conjugated, 1:200 dilution of anti-mouse Cy5 conjugated and 1:200 dilution of anti-rat Texas Red conjugated antibodies. All the antibodies were diluted in 1X PBS; 0.3% Triton X100. The incubation with the secondary antibodies was done for 2 hours at room temperature. The tissue was washed three times with 1X PBS; 0.3% Triton X100 followed by a brief immersion in 1X PBS. The eye discs were separated from their brains and mouth hook and mounted in 0.4% of n-propyll gallate in 1X PBS and Glycerol (0.0825g of n-propyl gallate; 15ml of 1XPBS and 5ml of Glycerol). For the triple staining with anti H3 rabbit generated, anti β-galactosidase and anti-elav rat generated the procedure was done as above but instead of

the anti Cyclin B, anti-phosphatase H3 1:1000 diluted in 1X PBS; 0.3% Triton X100 was added.

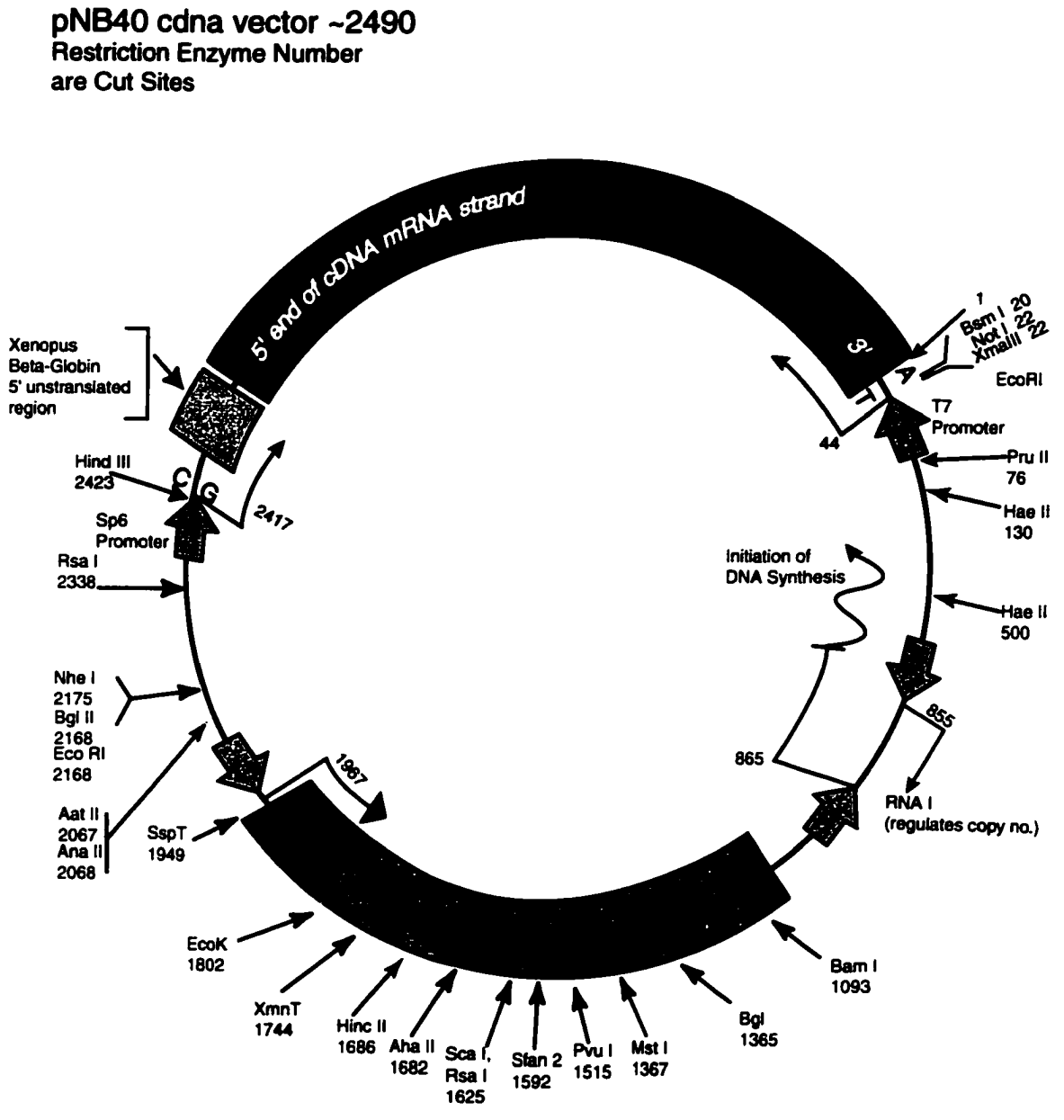
Scanning Electron Microscopy. The flies were anesthetized in CO_2 and dehydrated in 25% ethanol, 50% ethanol, 70% ethanol, 80% ethanol, 95% ethanol, 100% ethanol and 100% ethanol. The flies were critical point dried in a Balzers CPD 030. They were mounted in a scanning electron microscope stub and incubated for 24 hours in 65°C. Thereafter, they were coated sputter in a Denton Vacuum Desk II with 20ng of gold palladium. The flies were observed and pictures were taken in a Zeiss DSM 940 scanning electron microscope.

Plastic Embedding of Fly heads for Thick sections. The flies were anesthetized and decapitated. A small incision was made in the posterior part of the head to help the solution to get inside of the head. The heads were fixed in 4% glutaraldehyde in 0.1M Na Cacodylate buffer overnight at 4°C. The tissue was washed three times in 0.1M Na Cacodylate buffer at room temperature. The head were post fixed in 2.0% osmium tetroxide in 0.1M Sodium Cacodylate buffer for 1 hour at room temperature. Thereafter, they were washed three times in 0.1M Sodium Cacodylate buffer for 5 minutes at room temperature. The heads were dehydrated in 30%, 50%, 70%, 80%, 95%, 100% and 100% Ethanol at room temperature for 10 minutes each. The tissue was transferred to 5% Ethanol; 95% Propylene Oxide, followed 100% Propylene Oxide for 10 minutes each at room temperature. This was followed by a solution of 1:1 Propylene Oxide: PolyBed Resin for two hours at room temperature with constant stirring. The heads were transferred to 100% PolyBed resin without the DMP30 catalyzer and incubated overnight at room temperature. The tissue was transferred to 100% PolyBed with the catalyzer

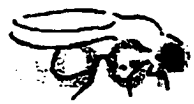
DMP30 and incubated at 45°C for 8-10 hours. The temperature was increased to 60°C and the incubation was continued for an additional 24-48 hours. One μm plastic sections were obtained using a LKB microtome and mounted on a subbed slides. The slides with the sections were warmed for 10 minutes at 70°C. Thereafter the sections were stained at 70°C for one hour 20 minutes with 2% toludine blue. The sections were rinsed with ddH_2O twice for 5 minutes each at room temperature. The tissue was observed in an axiopan microscope.

Appendix

Appendix 1: *fzr* cDNA Construct



Appendix 2



Drosophila Genome Projects Blast Searches

Job started at: 11:22:20 (Pacific time)

```
OK
BLASTN 2.0MP-WashU [13-Sep-2001] [sol8-ultra-ILP32F64 22:11:38 13-Sep-2001]

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Reference:  Gish, W. (1996-2001) http://blast.wustl.edu

Notice:  this program and its default parameter settings are optimized to find
nearly identical sequences rapidly.  To identify weak similarities encoded in
nucleic acid, use BLASTX, TBLASTN or TBLASTX.

Query=  tmp, 410 bases, 26A45217 checksum.
        (410 letters)

Database:  /data/blast/db/na_all.dros
           333,572 sequences; 565,431,042 total letters.
Searching....10....20....30....40....50....60....70....80....90....100% done

                                                                    Smallest
                                                                      Sum
                                                             High  Probability
Sequences producing High-scoring Segment Pairs:              Score   P(N)       N

GH18142.5prime AI387581 [similar by BLASTN (1.2e-110) to ...  1127  5.3e-45    1
rap|FBgn0[illegible]|CT[illegible]|FBan0003000 GO:[cell cycle regula...  1127  1.1e-44    1
GH12[illegible].3prime AI455119 [similar by BLASTN (2.2e-93) to f...  1115  1.4e-44    1
gb|Y14162|DMRNAFRP Drosophila melanogaster mRNA for Fizzy...  1119  2.8e-44    1
AC003715 : Drosophila melanogaster X BAC RP98-17H6 (Roswe...  1127  4.2e-44    1
gadfly|SEQ:AE003431|gb|AE003431|arm:X [4198619,4510082] e...  1127  4.2e-44    1
AI121813 : Drosophila melanogaster chromosome X clone BAC...  1077  1.4e-41    1
AI121865 : Drosophila melanogaster chromosome X clone BAC...   666  8.8e-23    1


>GH18141.5prime AI387581 [similar by BLASTN (1.2e-110) to fzr "FBan0003000
          "cell cycle regulator"  located on: X 4C7-4C8;" 04/14/2001]
        Length = 530

  Minus Strand HSPs:

 Score = 1127 (175.1 bits), Expect = 5.3e-45, P = 5.3e-45
 Identities = 271/345 (78%), Positives = 271/345 (78%), Strand = Minus / Plus

Query:   409 GGNCCCNGNCTCTCAGGTTTNCAATTTGCCNTGGTCCAAGCACTCCTCTGGACTTGGTTT 350
            || | | | ||| |||||||| |||| || | ||||||||||||||||| |||  |||| |
Sbjct:   152 GGACACGGGCTCGCAGGTTTGCAATCTGGCCTGGTCCAAGCACTCCTC-GGAGCTGGTCT 210
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Query:   349 CCACGCGCGNTTANTCGCAGANCCANATNNTCGCNTGGAANTNTCCCTCCGTNACTCCAA 290
             |||||| ||  || ||||||| ||| ||  |||  ||||| | ||||||| | || | ||
Sbjct:   211 CCACGCACGGCTACTCGCAGAACCAGATACTCGTGTGGAAATATCCCTCCCTGACGC-AA 269

Query:   289 GTGNCCAAGCT-ACGNCCCATTCGTATNNTGTCCTCNATNTGNCGATNAGTCCCGATGNC 231
             ||| ||||||| ||| ||||||||||  ||| ||| || || || | ||||||||||
Sbjct:   270 GTGGCCAAGCTGACGGGCCATTCGTATCGTGTGCTCTATCTGGCGCTGAGTCCCGATGGT 329

Query:   230 NANNNTATTGCNNCGCGGCGCCGAATACGATNCNNNNNGATNNTGTAACGTATTCAGCAA 171
               |   |||||   || |||||||   ||||  |     |||  || ||||||||||||||
Sbjct:   330 GAGGCTATTGTTACG-GGCGCCGGCGACGAGACGCTGCGATTTTGGAACGTATTCAGCAA 388

Query:   170 GGCGCGCAGTCAGAAGGAGAACAAGTCCGTTCTGAATCTGTNTNCAAATATCAGATANGG 111
             ||||||||||||||||||||||||||||||||||||||||||| | | ||||||||||| ||
Sbjct:   389 GGCGCGCAGTCAGAAGGAGAACAAGTCCGTTCTGAATCTGTTTGCCAATATCAGATAAGG 448

Query:   110 NCAATANCTCCAAGCGAGCCANGACTGAGCGAGCGNCAAAGGCNA 66
             ||||| |||||||||||| | ||||||||||||| ||||||| |
Sbjct:   449 ACAATAACTCCAAGCGAGCGAAGACTGAGCGAGCGCCAAAGGCAA 493
```

>rap|FBan0003000|CT12109|FBan0003000 GO:[cell cycle regulator (GO:0003750); degradation of cyclin (GO:0008054)] located on: X 4C7-4C8; [cDNA sequence

Length = 2645

Minus Strand HSPs:

Score = 1127 (175.1 bits), Expect = 1.1e-44, P = 1.1e-44
Identities = 271/345 (78%), Positives = 271/345 (78%), Strand = Minus / Plus

```
Query:   409 GGNCCCNGNCTCTCAGGTTTNCAATTTGCCNTGGTCCAAGCACTCCTCTGGACTTGGTTT 350
             || | | | ||| ||||||| |||| || | ||||||||||||||||| |||  |||| |
Sbjct:  1640 GGACACGGGCTCGCAGGTTTGCAATCTGGCCTGGTCCAAGCACTCCTC-GGAGCTGGTCT 1698

Query:   349 CCACGCGCGNTTANTCGCAGANCCANATNNTCGCNTGGAANTNTCCCTCCGTNACTCCAA 290
             |||||| ||  || ||||||| ||| ||  |||  ||||| | ||||||| | || | ||
Sbjct:  1699 CCACGCACGGCTACTCGCAGAACCAGATACTCGTGTGGAAATATCCCTCCCTGACGC-AA 1757

Query:   289 GTGNCCAAGCT-ACGNCCCATTCGTATNNTGTCCTCNATNTGNCGATNAGTCCCGATGNC 231
             ||| ||||||| ||| ||||||||||  ||| ||| || || || | ||||||||||
Sbjct:  1758 GTGGCCAAGCTGACGGGCCATTCGTATCGTGTGCTCTATCTGGCGCTGAGTCCCGATGGT 1817

Query:   230 NANNNTATTGCNNCGCGGCGCCGAATACGATNCNNNNNGATNNTGTAACGTATTCAGCAA 171
               |   |||||   || |||||||   ||||  |     |||  || ||||||||||||||
Sbjct:  1818 GAGGCTATTGTTACG-GGCGCCGGCGACGAGACGCTGCGATTTTGGAACGTATTCAGCAA 1876

Query:   170 GGCGCGCAGTCAGAAGGAGAACAAGTCCGTTCTGAATCTGTNTNCAAATATCAGATANGG 111
             ||||||||||||||||||||||||||||||||||||||||||| | | ||||||||||| ||
Sbjct:  1877 GGCGCGCAGTCAGAAGGAGAACAAGTCCGTTCTGAATCTGTTTGCCAATATCAGATAAGG 1936

Query:   110 NCAATANCTCCAAGCGAGCCANGACTGAGCGAGCGNCAAAGGCNA 66
             ||||| |||||||||||| | ||||||||||||| ||||||| |
Sbjct:  1937 ACAATAACTCCAAGCGAGCGAAGACTGAGCGAGCGCCAAAGGCAA 1981
```

>LD01270.3prime AI455119 [similar by BLASTN (2.2e-93) to fzr "FBan0003000 "cell cycle regulator" located on: X 4C7-4C8;" 04/16/2001]

Length = 498

Plus Strand HSPs:

Score = 1118 (173.8 bits), Expect = 1.4e-44, P = 1.4e-44

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 Identities = 270/345 (78%), Positives = 270/345 (78%), Strand = Plus / Plus

Query:    66 TNGCCTTTGNCGCTCGCTCAGTCNTGGCTCGCTTGGAGNTATTGNCCNTATCTGATATTT 125
             | ||||||| |||||||||||| | |||||||||||| ||||| || ||||||||||
Sbjct:    84 TTGCCTTTGGCGCTCGCTCAGTCTTCGCTCGCTTGGAGTTATTGTCCTTATCTGATATTG 143

Query:   126 GNANACAGATTCAGAACGGACTTGTTCTCCTTCTGACTGCGCGCCTTGCTGAATACGTTA 185
             | | ||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   144 GCAAACAGATTCAGAACGGACTTGTTCTCCTTCTGACTGCGCGCCTTGCTGAATACGTTC 203

Query:   186 CANNATCNNNNNGNATCGTATTCGGCGCCGCGNNGCAATANNNTNGNCATCGGGACTNAT 245
             || |||     | |||| ||||||| ||   |||||   |  |||||||||| |
Sbjct:   204 CAAAATCGCAGCGTCTCGTCGCCGGCGCC-CGTAACAATAGCCTCACCATCGGGACTCAG 262

Query:   246 CGNCANATNGAGGACANNATACGAATGGGNCGT-AGCTTGGNCACTTGGAGTNACGGAGG 304
             || || || ||| ||| ||||||||| ||| ||||||| |||||| || | |||||
Sbjct:   263 CGCCAGATAGAGCACACGATACGAATGGCCCGTCAGCTTGGCCACTTGC-GTCAGGGAGG 321

Query:   305 GANANTTCCANGCGANNATNTGGNTCTGCGANTAANCGCGCGTGGAAACCAAGTCCAGAG 364
             || | ||||| ||| || ||| ||||||| ||  || ||||||| |||| ||| |||
Sbjct:   322 GATATTTCCACACGAGTATCTGGTTCTGCGATTATCCGTGCGTGGAGACCAGCTCC-GAG 380

Query:   365 GAGTGCTTGGACCANGGCAAATTGNAAACCTGAGAGNCNGGGNCC 409
             |||||||||||||| | || |||| ||||||| ||  | | | ||
Sbjct:   381 GAGTGCTTGGACCAGGCCAGATTGCAAACCTGCGATCCCGTGTCC 425


>gb|Y14161|DMRNAFRP Drosophila melanogaster mRNA for Fizzy-related protein.
          CDS:468..1904.
        Length = 2605

  Minus Strand HSPs:

 Score = 1118 (173.8 bits), Expect = 2.8e-44, P = 2.8e-44
 Identities = 270/345 (78%), Positives = 270/345 (78%), Strand = Minus / Plus

Query:   409 GGNCCCNGNCTCTCAGGTTTNCAATTTGCCNTGGTCCAAGCACTCCTCTGGACTTGGTTT 350
             || | | | ||| ||||||| |||| || | |||||||||||||||| |||  |||| |
Sbjct:  1610 GGACACGGGCTCGCAGGTTTGCAATCTGGCCTGGTCCAAGCACTCCTC-GGAGCTGGTCT 1668

Query:   349 CCACGCGCGNTTANTCGCAGANCCANATNNTCGCNTGGAANTNTCCCTCCGTNACTCCAA 290
             |||||| ||  || ||||||| ||| ||  |||  ||||| | ||||||| | || | ||
Sbjct:  1669 CCACGCACGGCTACTCGCAGAACCAGATACTCGTGTGGAAATATCCCTCCCTGACGC-AA 1727

Query:   289 GTGNCCAAGCT-ACGNCCCATTCGTATNNTGTCCTCNATNTGNCGATNAGTCCCGATGNC 231
             ||| |||||||  |||  |||||||||||  ||| ||| |  || || | ||||||||||
Sbjct:  1728 GTGGCCAAGCTGACGGGCCATTCGTATCGTGTGCTCTACCTGGCGCTGAGTCCCGATGGT 1787

Query:   230 NANNNTATTGCNNCGCGGCGCCGAATACGATNCNNNNNGATNNTGTAACGTATTCAGCAA 171
              |   |||||   || |||||||   ||||  |      |||  || ||||||||||||||
Sbjct:  1788 GAGGCTATTGTTACG-GGCGCCGGCGACGAGACGCTGCGATTTTGGAACGTATTCAGCAA 1846

Query:   170 GGCGCGCAGTCAGAAGGAGAACAAGTCCGTTCTGAATCTGTNTNCAAATATCAGATANGG 111
             ||||||||||||||||||||||||||||||||||||||||||  | | ||||||||| ||
Sbjct:  1847 GGCGCGCAGTCAGAAGGAGAACAAGTCCGTTCTGAATCTGTTTGCCAATATCAGATAAGG 1906

Query:   110 NCAATANCTCCAAGCGAGCCANGACTGAGCGAGCGNCAAAGGCNA 66
              ||||| |||||||||||| | |||||||||||||| ||||||| |
Sbjct:  1907 ACAATAACTCCAAGCGAGCGAAGACTGAGCGAGCGCCAAAGGCAA 1951


AC 013715 : Drosophila melanogaster X BAC RP98-17H6 (Roswell Park Cancer
          Institute Drosophila BAC Library) complete sequence.  Last updated
          01-JAN-2002
```

```
        Length = 160,622

  Plus Strand HSPs:

 Score = 1127 (175.1 bits), Expect = 4.2e-44, P = 4.2e-44
 Identities = 271/345 (78%), Positives = 271/345 (78%), Strand = Plus / Plus

Query:     66 TNGCCTTTGNCGCTCGCTCAGTCNTGGCTCGCTTGGAGNTATTGNCCNTATCTGATATTT 125
              | ||||||| |||||||||||| | |||||||||||| ||||| || ||||||||||
Sbjct: 37807 TTGCCTTTGGCGCTCGCTCAGTCTTCGCTCGCTTGGAGTTATTGTCCTTATCTGATATTG 37866

Query:    126 GNANACAGATTCAGAACGGACTTGTTCTCCTTCTGACTGCGCGCCTTGCTGAATACGTTA 185
              | | ||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 37867 GCAAACAGATTCAGAACGGACTTGTTCTCCTTCTGACTGCGCGCCTTGCTGAATACGTTC 37926

Query:    186 CANNATCNNNNNGNATCGTATTCGGCGCCGCGNNGCAATANNNTNGNCATCGGGACTNAT 245
              ||  |||     | ||||  ||||||| ||   |||||  |   |||||||||| |
Sbjct: 37927 CAAAATCGCAGCGTCTCGTCGCCGGCGCC-CGTAACAATAGCCTCACCATCGGGACTCAG 37985

Query:    246 CGNCANATNGAGGACANNATACGAATGGGNCGT-AGCTTGGNCACTTGGAGTNACGGAGG 304
              || || || ||| |||  |||||||||| ||| ||||||| ||||||  || | |||||
Sbjct: 37986 CGCCAGATAGAGCACACGATACGAATGGCCCGTCAGCTTGGCCACTTGC-GTCAGGGAGG 38044

Query:    305 GANANTTCCANGCGANNATNTGGNTCTGCGANTAANCGCGCGTGGAAACCAAGTCCAGAG 364
              || | |||||  |||  || ||| ||||||| ||  || ||||||| ||||  ||| |||
Sbjct: 38045 GATATTTCCACACGAGTATCTGGTTCTGCGAGTAGCCGTGCGTGGAGACCAGCTCC-GAG 38103

Query:    365 GAGTGCTTGGACCANGGCAAATTGNAAACCTGAGAGNCNGGGNCC 409
              |||||||||||||| | || |||| ||||||| ||| | | | ||
Sbjct: 38104 GAGTGCTTGGACCAGGCCAGATTGCAAACCTGCGAGCCCGTGTCC 38148


>gadfly|SEG:AE003431|gb|AE003431|arm:X [4198619,4510082] estimated-cyto:4C1-4C8
          gadfly-seqname:AE003431.2
        Length = 311,464

  Plus Strand HSPs:

 Score = 1127 (175.1 bits), Expect = 4.2e-44, P = 4.2e-44
 Identities = 271/345 (78%), Positives = 271/345 (78%), Strand = Plus / Plus

Query:     66 TNGCCTTTGNCGCTCGCTCAGTCNTGGCTCGCTTGGAGNTATTGNCCNTATCTGATATTT 125
              | ||||||| |||||||||||| | |||||||||||| ||||| || ||||||||||
Sbjct: 278861 TTGCCTTTGGCGCTCGCTCAGTCTTCGCTCGCTTGGAGTTATTGTCCTTATCTGATATTG 278920

Query:    126 GNANACAGATTCAGAACGGACTTGTTCTCCTTCTGACTGCGCGCCTTGCTGAATACGTTA 185
              | | ||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 278921 GCAAACAGATTCAGAACGGACTTGTTCTCCTTCTGACTGCGCGCCTTGCTGAATACGTTC 278980

Query:    186 CANNATCNNNNNGNATCGTATTCGGCGCCGCGNNGCAATANNNTNGNCATCGGGACTNAT 245
              ||  |||     | ||||  ||||||| ||   |||||  |   |||||||||| |
Sbjct: 278981 CAAAATCGCAGCGTCTCGTCGCCGGCGCC-CGTAACAATAGCCTCACCATCGGGACTCAG 279039

Query:    246 CGNCANATNGAGGACANNATACGAATGGGNCGT-AGCTTGGNCACTTGGAGTNACGGAGG 304
              || || || ||| |||  |||||||||| ||| ||||||| ||||||  || | |||||
Sbjct: 279040 CGCCAGATAGAGCACACGATACGAATGGCCCGTCAGCTTGGCCACTTGC-GTCAGGGAGG 279098

Query:    305 GANANTTCCANGCGANNATNTGGNTCTGCGANTAANCGCGCGTGGAAACCAAGTCCAGAG 364
              || | |||||  |||  || ||| ||||||| ||  || ||||||| ||||  ||| |||
Sbjct: 279099 GATATTTCCACACGAGTATCTGGTTCTGCGAGTAGCCGTGCGTGGAGACCAGCTCC-GAG 279157

Query:    365 GAGTGCTTGGACCANGGCAAATTGNAAACCTGAGAGNCNGGGNCC 409
              |||||||||||||| | || |||| ||||||| ||| | | | ||
Sbjct: 279158 GAGTGCTTGGACCAGGCCAGATTGCAAACCTGCGAGCCCGTGTCC 279202
```

AL111611 : Drosophila melanogaster chromosome X clone BACR2I23 map 4D strain y; cn bw sp, *** SEQUENCING IN PROGRESS ***, in unordered pieces.
Last updated 11-OCT-1999
Length = 96,924

Plus Strand HSPs:

Score = 1071 (166.7 bits), Expect = 1.4e-41, P = 1.4e-41
Identities = 265/343 (77%), Positives = 265/343 (77%), Strand = Plus / Plus

```
Query:    66 TNGCCTTTGNCGCTCGCTCAGTCNTGGCTCGCTTGGAGNTATTGNCCNTATCTGATATTT 125
             | ||||||| |||||||||||||| | |||||||||||| ||||| || ||||||||||
Sbjct: 74058 TTGCCTTTGGCGCTCGCTCAGTCTTCGCTCGCTTGGAGTTATTGTCCTTATCTGATATTG 74117

Query:   126 GNANACAGATTCAGAACGGACTTGTTCTCCTTCTGACTGCGCGCCTTGCTGAATACGTTA 185
             | | ||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 74118 GCAAACAGATTCAGAACGGACTTGTTCTCCTTCTGACTGCGCGCCTTGCTGAATACGTTC 74177

Query:   186 CANNATCNNNNNGNATCGTATTCGGCGCCGCGNNGCAATANNNTNGNCATCGGGACTNAT 245
             ||  |||     | ||||  ||||||| ||  |||||  |  ||||||||| |
Sbjct: 74178 CAAAATCGCAGCGTCTCGTCGCCGGCGCC-CGTAACAATAGCCTCACCATCGGGACTCAG 74236

Query:   246 CGNCANATNGAGGACANNATACGAATGGGNCGT-AGCTTGGNCACTTGGAGTNACGGAGG 304
             || || || ||| |||  |||||||||  ||| ||||||| ||||||  || | ||| |
Sbjct: 74237 CGCCAGATAGAGCACACGATACGAATGGCCCGTCAGCTTGGCCACTTGC-GTCAGGGAAG 74295

Query:   305 GANANTTCCANGCGANNATNTGGNTCTGCGANTAANCGCGCGTGGAAACCAAGTCCAGAG 364
             || | |||||  |||  || ||| ||||||| ||  || ||||||| | ||  ||| |||
Sbjct: 74296 GATATTTCCACACGAGTATCTGGTTCTGCGAGTANGCGTGCGTGGAGAACAGCTCC-GAG 74354

Query:   365 GAGTGCT-TGGACCANGGCAAATTGNAAACCTGAGAGNCNGGG 406
             ||||||| ||||| | |||| |||| | ||||| ||  | | |
Sbjct: 74355 GAGTGCTNTGGAC-AGGGCAGATTGCANACCTGCGAAGCCGTG 74396
```

AL111653 : Drosophila melanogaster chromosome X clone BACR3H4 map 4B-C strain
Last updated 15-OCT-1999
Length = 89,247

Plus Strand HSPs:

Score = 347 (58.1 bits), Expect = 7.2e-09, P = 7.2e-09
Identities = 85/110 (77%), Positives = 85/110 (77%), Strand = Plus / Plus

```
Query:   300 GGAGGGANANTTCCANGCGANNATNTGGNTCTGCGANTAANCGCGCGTGGAAACCAAGTC 359
             ||||||| | |||||  |||  || ||| ||||||| ||  || ||||||| ||||  ||
Sbjct:     2 GGAGGGATATTTCCACACGAGTATCTGGTTCTGCGAGTAGCCGTGCGTGGAGACCAGCTC 61

Query:   360 CAGAGGAGTGCTTGGACCANGGCAAATTGNAAACCTGAGAGNCNGGGNCC 409
             : |||||||||||||||| | || |||| ||||||| ||| | | | ||
Sbjct:    62 C-GAGGAGTGCTTGGACCAGGCCAGATTGCAAACCTGCGAGCCCGTGTCC 110
```

Minus Strand HSPs:

Score = 655 (104.3 bits), Expect = 8.8e-23, P = 8.8e-23
Identities = 157/201 (78%), Positives = 157/201 (78%), Strand = Minus / Plus

```
Query:   266 TATNNTGTCCTCNATNTGNCGATNAGTCCCGATGNCNANNNTATTGCNNCGCGGCGCCGA 207
             |||  ||| ||| || || || | |||||||||  |  |||||  || |||||||
Sbjct: 56437 TATCGTGTGCTCTATCTGGCGCTGAGTCCCGATGGTGAGGCTATTGTTACG-GGCGCCGG 56495

Query:   206 ATACGATNCNNNNNGATNNTGTAACGTATTCAGCAAGGCGCGCAGTCAGAAGGAGAACAA 147
```

AL121511 : Drosophila melanogaster chromosome X clone BACR2I23 map 4D strain y; cn bw sp, *** SEQUENCING IN PROGRESS ***, in unordered pieces. Last updated 11-OCT-1999
Length = 96,924

Plus Strand HSPs:

Score = 1071 (166.7 bits), Expect = 1.4e-41, P = 1.4e-41
Identities = 265/343 (77%), Positives = 265/343 (77%), Strand = Plus / Plus

```
Query:    66 TNGCCTTTGNCGCTCGCTCAGTCNTGGCTCGCTTGGAGNTATTGNCCNTATCTGATATTT 125
             | ||||||| |||||||||||||| | |||||||||||| ||||| || ||||||||||
Sbjct: 74058 TTGCCTTTGGCGCTCGCTCAGTCTTCGCTCGCTTGGAGTTATTGTCCTTATCTGATATTG 74117

Query:   126 GNANACAGATTCAGAACGGACTTGTTCTCCTTCTGACTGCGCGCCTTGCTGAATACGTTA 185
             | | ||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 74118 GCAAACAGATTCAGAACGGACTTGTTCTCCTTCTGACTGCGCGCCTTGCTGAATACGTTC 74177

Query:   186 CANNATCNNNNNGNATCGTATTCGGCGCCGCGNNGCAATANNNTNGNCATCGGGACTNAT 245
             ||  |||     |  ||||  ||||||| ||   |||||   |   |||||'|||| |
Sbjct: 74178 CAAAATCGCAGCGTCTCGTCGCCGGCGCC-CGTAACAATAGCCTCACCATCGGGACTCAG 74236

Query:   246 CGNCANATNGAGGACANNATACGAATGGGNCGT-AGCTTGGNCACTTGGAGTNACGGAGG 304
             || || || ||| |||  |||||||||| ||| ||||||| |||||  || | ||| |
Sbjct: 74237 CGCCAGATAGAGCACACGATACGAATGGCCCGTCAGCTTGGCCACTTGC-GTCAGGGAAG 74295

Query:   305 GANANTTCCANGCGANNATNTGGNTCTGCGANTAANCGCGCGTGGAAACCAAGTCCAGAG 364
             || | |||||  |||  || ||| ||||||| ||  || ||||||| | ||  ||| |||
Sbjct: 74296 GATATTTCCACACGAGTATCTGGTTCTGCGAGTANGCGTGCGTGGAGAACAGCTCC-GAG 74354

Query:   365 GAGTGCT-TGGACCANGGCAAATTGNAAACCTGAGAGNCNGGG 406
             ||||||| ||||| | |||| |||| | ||||| ||  | | |
Sbjct: 74355 GAGTGCTNTGGAC-AGGGCAGATTGCANACCTGCGAAGCCGTG 74396
```

AL121913 : Drosophila melanogaster chromosome X clone BACR3H4 map 4B-C strain
Last updated 15-OCT-1999
Length = 89,247

Plus Strand HSPs:

Score = 347 (58.1 bits), Expect = 7.2e-09, P = 7.2e-09
Identities = 85/110 (77%), Positives = 85/110 (77%), Strand = Plus / Plus

```
Query:   300 GGAGGGANANTTCCANGCGANNATNTGGNTCTGCGANTAANCGCGCGTGGAAACCAAGTC 359
             ||||||| | |||||  |||  || ||| ||||||| ||  || ||||||| ||||  ||
Sbjct:     2 GGAGGGATATTTCCACACGAGTATCTGGTTCTGCGAGTAGCCGTGCGTGGAGACCAGCTC 61

Query:   360 CAGAGGAGTGCTTGGACCANGGCAAATTGNAAACCTGAGAGNCNGGGNCC 409
             : |||||||||||||||| | || |||| ||||||| ||| | | | ||
Sbjct:    62 C-GAGGAGTGCTTGGACCAGGCCAGATTGCAAACCTGCGAGCCCGTGTCC 110
```

Minus Strand HSPs:

Score = 655 (104.3 bits), Expect = 8.8e-23, P = 8.8e-23
Identities = 157/201 (78%), Positives = 157/201 (78%), Strand = Minus / Plus

```
Query:   266 TATNNTGTCCTCNATNTGNCGATNAGTCCCGATGNCNANNNTATTGCNNCGCGGCGCCGA 207
             |||  ||| ||| || || || | |||||||||   |   |||||   || |||||||
Sbjct: 56437 TATCGTGTGCTCTATCTGGCGCTGAGTCCCGATGGTGAGGCTATTGTTACG-GGCGCCGG 56495

Query:   206 ATACGATNCNNNNNGATNNTGTAACGTATTCAGCAAGGCGCGCAGTCAGAAGGAGAACAA 147
```

```
                 ||||  |     |||  || | ||||||||||||||||||||||||||||||||||||
Sbjct: 56496 CGACGAGACGCTGCGATTTTGGA-CGTATTCAGCAAGGCGCGCAGTCAGAAGGAGAACAA 56554

Query:   146 GTCCGTTCTGAATCTGTNTNCAAATATCAGATANGGNCAATANCTCCAAGCGAGCCANGA 87
             |||||||||||||||| | |  |||||||||  || ||||| ||||||||||| | ||
Sbjct: 56555 GTCCGTTCTGAATCTGTTTGCCGATATCAGATTAGGACAATAACTCCAAGCGAGCGAAGA 56614

Query:    86 CTGAGCGAGCGNCAAAGGCNA 66
             |||||||||||| ||||||| |
Sbjct: 56615 CTGAGCGAGCGCCAAAGGCAA 56635


Parameters:
  B=50
  V=100
  sort_by_pvalue
  filter=dust
  sump

  ctxfactor=2.00
  E=10

  Query                          -----  As Used  -----     -----  Computed  ----
  Strand MatID Matrix name       Lambda     K       H      Lambda     K       H
   +1      0   +5,-4             0.192   0.173   0.357     same     same    same
               Q=10,R=10         0.104   0.0151  0.0600     n/a      n/a     n/a
   -1      0   +5,-4             0.192   0.173   0.357     same     same    same
               Q=10,R=10         0.104   0.0151  0.0600     n/a      n/a     n/a

  Query
  Strand MatID  Length  Eff.Length     E     S  W   T  X   E2       S2
   +1      0      410         349     10.   175 11  n/a 73  0.024     76
                                                      83  0.023    109
   -1      0      410         349     10.   175 11  n/a 73  0.024     76
                                                      83  0.023    109

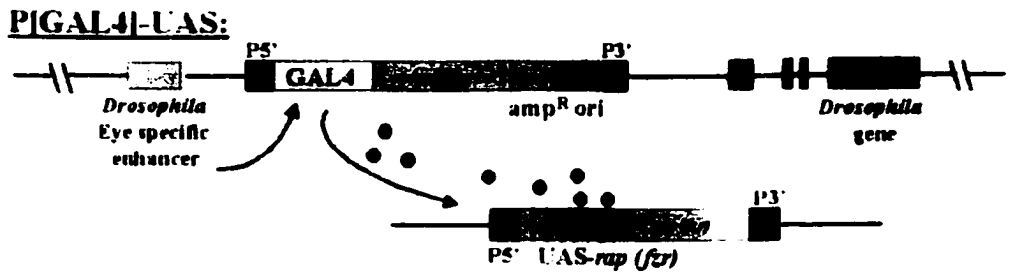

Statistics:

  Database:  /data/blast/db/na_all.dros
   Title:  /data/blast/db/na_all.dros
   Posted:  8:11:04 PM PST Mar 16, 2002
   Format:  BLAST-1.4
   # of letters in database:  565,431,042
   # of sequences in database:  333,572
   # of database sequences satisfying E:  8
  No. of states in DFA:  164 (164 KB)
  Total size of DFA:  172 KB (2053 KB)
  Time to generate neighborhood:  0.03u 0.13s 0.16t  Elapsed: 00:00:00
  No. of threads or processors used:  4
  Search cpu time:  20.99u 4.13s 25.12t  Elapsed: 00:00:08
  Total cpu time:  21.07u 4.32s 25.39t  Elapsed: 00:00:08
  Start:  Wed Mar 20 11:21:53 2002   End:  Wed Mar 20 11:22:01 2002
```

BDGP Home | Drosophila BLAST

Appendix 3

Targeted Expression of genes in Drosophila

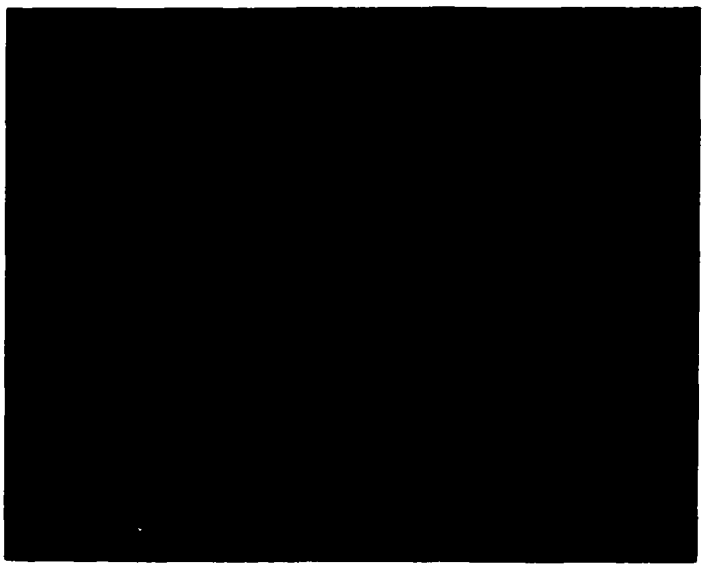


A schematic diagram of the method for targeted expression of *rap (fzr)* using the GAL4-UAS system of Brand and Perrimon (1993). When the flies carrying the *UAS-rap (fzr)* are crossed to the *ey-GAL4* flies, eye specific expression of *rap (fzr)* is seen the F_1.

Appendix 4: List of Gal4 drivers Used with the *UAS-fzr* line

Gal 4 Drivers	Phenotypic Effect	Gal 4 Drivers	Phenotypic Effect
atonal (*ato*)	None	*en 2.4*	None
hairy (*h*)	Lethality	ay (25)	None
lozenge (*lz*)	None	ay (17B)	None
elav (*elav*)	Rough eyes	*derailed* (*drl*)	None
armadillo (*arm*)	None	*Distal-less* (*dll*)	None
paired (*prd*)	None	*dachshund*	Rough eyes, Tumors in the legs
rhomboid (*ve*)	None	*en 2.4*	None
Actin (5C act5c)	None		
		eyeless (*ey*)	Rough eyes, Ectopic antennas, Tumor development, Ectopic eyes.
ninaE	None		
decapentaplegic (*dpp*)	None		
		en 2.4	None
GawB (*34B*)	None		

Appendix 5



The *UAS-lacZ: ey-GAL4* stained with anti β-galactosidase to study the expression pattern of the *ey* gene.

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