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STUDIES ON PATTERN FORMATION AND CELL FATE DETERMINATION IN THE *DROSOPHILA* COMPOUND EYE: THE ROLE OF THE *rugose* GENE

by

HODA K. SHAMLOULA

A dissertation submitted to the Graduate Faculty in Biology

in partial fulfillment of the requirements for the degree of

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1996

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Abstract

STUDIES ON PATTERN FORMATION AND CELL FATE DETERMINATION IN THE *DROSOPHILA* COMPOUND EYE: THE ROLE OF THE *rugose* GENE

by

Hoda K. Shamloula

Advisor: Professor Tadmiri Venkatesh

Understanding how complex and highly organized cellular patterns are formed is a fundamental question in developmental biology. The *Drosophila* compound eye is a highly precise cellular pattern readily amenable to genetic analysis. During the development of the *Drosophila* compound eye, cell-cell interactions play a critical role in the sequential determination of cell fates leading to a precise cellular patterns. The *Drosophila* compound eye consists of eight hundred unit-eye called ommatidia which are arranged in a regular symmetrical array. Each ommatidium is comprised of an invariant number of neural and nonneural cells arranged in a precise manner. The development of the retina begins with the differentiation of the neural photoreceptor

cell R8 which orchestrates the differentiation of the other photoreceptors via inductive and inhibitory cues. Subsequently the nonneural cone and pigment cells are specified by unknown mechanisms. Cone cells and pigment cells are involved in the formation of the lens and the lattice between the ommatidia establishing specific cell-cell contacts needed for a functional retina. The cone cells, in addition to secreting the lens, are important in the formation of the retinal pattern and the fenestrated basement membrane that shapes and supports the entire retina. To study the cellular and molecular interactions that direct the retinal pattern formation, I have isolated and characterized mutations in the *rugose* gene. My genetic and phenotypic studies show that mutations at the *rg* locus (4E-F) results in a rough eye phenotype. Cobalt sulfide and electron microscope studies have shown that *rg* is required for proper differentiation of cone and pigment cells and for the formation of the fenestrated basement membrane. Genetic interaction studies show that *rg* may function in the EGF receptor mediated signal transduction pathway. The abnormal *rg* wing phenotype, that shows incomplete wing venation and multiple cross veins, supports the notion that *rg* may function through EGFr pathway. These results suggest that *rg* may be an important effector of the cone cell differentiation and its function may be mediated through the EGF receptor mediated signal cascade. A putative genomic DNA clone that encodes the *rg* gene has been isolated by chromosomal walking.

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CHAPTER I

INTRODUCTION

During the development of multicellular organisms a single cell divides and gives rise to a variety of cell types that are often highly specialized, distributed in unique patterns and carry out specific functions. So far, very little is known as to by what mechanisms these cells differentiate and acquire their specific fates. Currently there are two models by which cell fates are thought to be determined. The cell lineage model where cell fate is determined by its ancestry, and the cellular induction model where cell-cell interactions occur through sending and responding to signals in the cells environment. These signals could be inductive or inhibitory. Induction occurs either by instructive signals that directly affect cell fates or by permissive signals that permit continued differentiation of cells whose fate has been determined. The *Drosophila* compound eye is an excellent system to study the role of cell-cell interactions in cell fate determination and pattern formation for several reasons. Mutations in the eye may not affect the viability of the fly, therefore many viable eye mutations are available. Both classical and molecular genetic manipulations are readily feasible. In the *Drosophila* eye, some of the sensory neurons which innervate mechano-sensory bristles are generated by cell lineage, while photoreceptor and pigment cells are generated by cell-cell interactions and positional cues (Ready et al. 1976; Lawrence and Green, 1979).

Recently clonal analysis suggests that cone cell determination is probably specified by cell-cell interactions (Wolff and Ready, 1991a).

Structure and Organization of The *Drosophila* Compound Eye

The *Drosophila* compound eye consists of nearly eight hundred repeated unit eyes or facets called "ommatidia" which are arranged in a regular symmetrical array. The dorsal and ventral halves of the compound eye are organized in a mirror image symmetry. The regular arrangement of the ommatidia is directed by the cellular lattice that surrounds and spaces the repetitive ommatidia. Each hexagonally shaped ommatidium includes eight photoreceptor neurons (R cells) and a precise number of nonneural accessory cells: four corneal lens secreting cone cells, six pigment cells and four cells forming a hair group [Fig. 1]. The cells in each ommatidium occupy specific positions resulting in the precise spatial pattern. The photoreceptors can be classified into three subgroups, R1-R6, R7 and R8 based on their positions, spectral sensitivity and synaptic connectivity (Braitenberg, 1967; Melamed and Trujillo-Cenoz, 1975). In each R cell, more than 90% of the photoreceptor's plasma membrane is multiply folded forming about 60,000 tightly packed microvilli called the rhabdomere that contain specific light sensitive photopigments (rhodopsin). The rhabdomeres of R1-R6 are almost of equal sizes and larger than the R7 and R8 rhabdomeres in cross sections. The photoreceptors R1-R6 extend their rhabdomeres the entire length of the retina and lie in an outer circle surrounding the central, distal R7 cell and the central, proximal

R8 cell (Wolff and Ready, 1993). The outer R1-R6 cells extend their axons to the first optic ganglion the lamina, while the central R7 and R8 axons synapse in the second optic ganglion the medulla. These axons terminate in the optic ganglia with a precise pattern that reflects the fly's visual field (Braitenberg, 1967).

The four cone cells occupy specific positions on top of the photoreceptor cells. The equatorial and polar cone cells form a contact while the anterior and posterior cone cells are separated. The anterior cone cell lies over R1-R3, the posterior cone cell extends above R4-R6, the polar cone cell covers R3 and R4, and the equatorial cone cell lies over R1, R6 and R7 [Fig. 2]. The pseudocone and the lens are acellular structures secreted by the cone and pigment cells. Cone cells are about 2μm thick with apical and basal surfaces. The cone cell apical surface, facing the floor of the pseudocone, contains small projections called papillae that secrete the lens. The basal surface facing the rhabdomeres extends thin fibers between the photoreceptors to the base of the retina. The feet of the cone cell fibers contact together (in a way that reverses the contact made by their cell bodies) below the end of the rhabdomeres to plug the ommatidial cavity at the floor of the retina (Cagan and Ready, 1989b). These cone cell feet contain ommochrome granules, and are often mistakenly called inner pigment cells (Wolff and Ready, 1993).

There are six pigment cells in each ommatidium. Two primary pigment cells that form the walls of the pseudocone chamber and wrap the four cone cells to separate them from the external lattice. The two primary pigment cells contain ommochrome pigments and are located towards the distal end of the photoreceptors. The primary

Figure 1. Schematic view of the *Drosophila* compound eye structure. (A) The compound eye consists of repeated units called ommatidia. The hexagonally shaped ommatidia are arranged in a regular symmetrical array that gives the eye its normal appearance. Each ommatidium contains eight photoreceptor cells (R cells), four cone cells, two primary pigment cells, three tertiary pigment cells, and a bristle group. (B) In a longitudinal section of a single unit eye, the distal R7 cell is positioned on top of the proximal R8 cell. These two central cells are surrounded by the six outer photoreceptors R1-R6. The primary pigment cells do not extend the length of the retina. (C) A cross section in the distal region of an ommatidium shows that the anterior and posterior cone cells are separated while the equatorial and polar cone cells contact. The four cone cells are wrapped by two primary pigment cells which are surrounded by the external hexagonal lattice. (D) A cross section in the proximal region shows seven photoreceptor cells surrounded by the lattice. The cone cells processes pass between the photoreceptors. (E) A cross section below R7's level shows R8 rhabdomere replacing R7's rhabdomere in the center. (B) bristle; (L) lens; (PS) pseudocone; (PPC) primary pigment cell; (CC) cone cell; (CCP) cone cell process; (SPC) secondary pigment cell; (BP) bristle process; (R7Rh) R7 rhabdomere; (R8Rh) R8 rhabdomere; (CCF) cone cell foot; (FM) fenestrated basement membrane; (TPC) tertiary pigment cell; (1-8) photoreceptors R1-R8

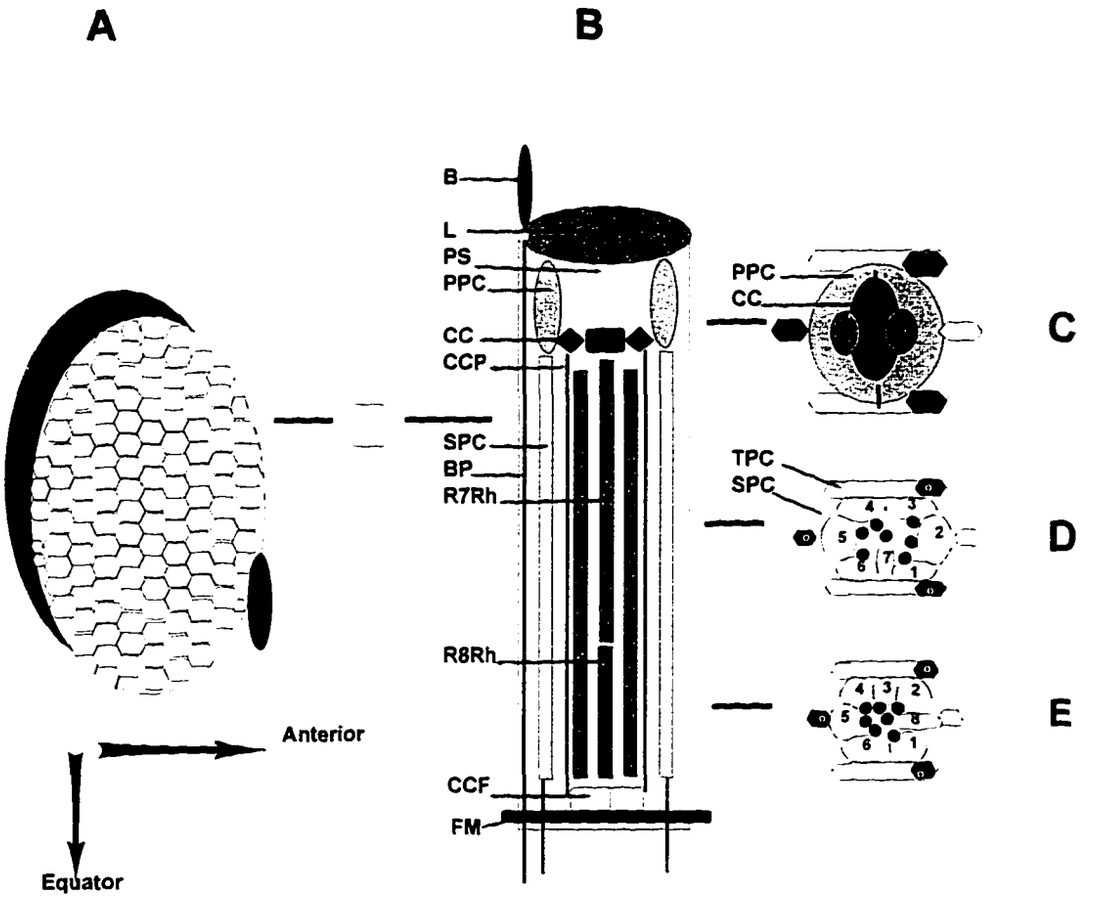


Figure I. Schematic view of the *Drosophila* compound eye structure.

pigment cells do not reach the floor of the retina. The outer lattice evenly spaces the ommatidia and apically isolates one from another. The lattice consists of shared pigment cells that contain both ommochrome and pteridine pigments. In the lattice three secondary pigment cells which contact two neighboring ommatidia and one tertiary pigment cell that contacts three ommatidia. The inner surface of the retina is supported and shaped by a specialized membrane called the fenestrated basement membrane [Fig. 1]. This membrane is formed from the flattened feet of the secondary and the tertiary pigment cells that contain actin fibers. The photoreceptor cells axons as well as the axon of the bristle neuron exit the retina to the optic lobe through reinforced eyelets in the fenestrated basement membrane (Cagan and Ready, 1989b). The mechanosensory bristle group projects at the anterior end of the horizontal secondary pigment cell. The bristle is formed from four cells, the tormogen, the tricogen, the thecogen and a sensory neuron. The tormogen and the tricogen secrete the socket and the bristle respectively, then they degenerate later in pupal stage (Perry, 1968, cited from Wolff and Ready, 1993).

Development of The Compound Eye

The *Drosophila* visual system develops from the eye-imaginal disc primordia and the optic lobe anlagen. These two regions have no direct contact till the third larval instar stage. During embryogenesis, the invagination of about twenty blastodermal cells forms the eye-antennal imaginal disc. The eye disc is a monolayer of

undifferentiated columnar epithelial cells covered with a peripodial membrane.

Development During The Third Larval Instar: Pattern formation begins during the mid-third larval instar. A dorso-ventral groove called the morphogenetic furrow (MF) sweeps from the posterior to the anterior leaving behind clusters of differentiating cells (Ready et al., 1976; Tomlinson, 1985; Tomlinson and Ready, 1987a,b). In the developing eye disc there are two bands of dividing cells. The first mitotic wave is located ahead of the furrow and the second mitotic wave behind the furrow. Anterior to the MF cells are undifferentiated and unpatterned. Posterior to the MF preclusters of five differentiating cells R2, R3, R4, R5 and R8 can be seen (Venkatesh et al., 1985). The premature cluster contains one or two undifferentiated cells called mystery cells that are eliminated later in development (Tomlinson and Ready, 1987a). As a result of the second mitotic wave, R1, R6 and R7 cells are added to the five cell precluster to form the eight cells of the mature cluster (Ready et al., 1976). Genetic mosaic studies during the 1970's clearly established that their are no strict lineage relationships between the cells of the developing eye disc (Ready et al., 1976; Lawrence and Green, 1979). Later, immunohistochemical studies following the appearance of neuronal antigens have shown that the photoreceptor cells differentiate in a sequential manner. These results led to the postulation of the inductive model for cellular pattern formation in the developing eye disc. In this model R8 cell initiates a series of inductive events that lead to the differentiation and the determination of the ommatidium (Tomlinson and Ready, 1987a,b). R8 cell differentiates first and induces R2 and R5 to differentiate followed by the differentiation of two neighboring cells into R3 and R4.

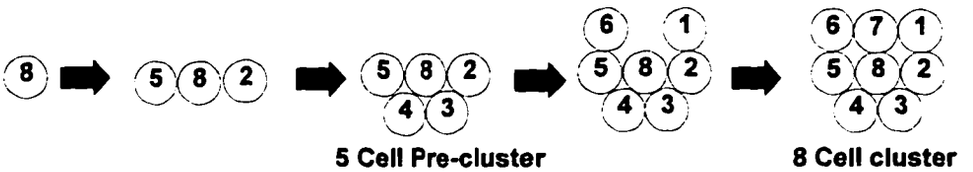
Subsequently R1 and R6 differentiate and the last cell to differentiate is the R7 cell [Fig. 2A]. The nuclei of the newly formed eight-cell cluster migrate to the apical surface of the third larval instar eye disc. By the end of the late third instar the eye disc everts leading the apical surface towards the outside of the fly. All eight R cells project their axons deeper in the tissue entering the optic stalk. As soon as the R7 nucleus migrates to the apical surface, two cone cells differentiate and their nuclei rise from the basal region and flank the photoreceptor cells cluster from the anterior and posterior to establish the two cone cell stage (Tomlinson, 1985). This is followed by the differentiation of two other cone cells that flank the cluster from the equatorial and the polar sides forming the four cone cell stage [Fig. 2B]. The four cone cells rise to the apical surface and cover the photoreceptor cells. This stage is the most advanced arrangement of the eye achieved before pupation.

Development During The Pupal Stage: Eye development during pupal stage can be divided into an early phase (0-60 hr of pupal formation) in which the cellular pattern of the retina is completed, and a late phase (60-160 hr) in which the final cellular structures and products are established (Cagan and Ready, 1989b). During the early pupal development at about 20-hr of pupation, two primary pigment cells spread around the anterior and posterior cone cells and at 30-hr they meet each other across the equatorial and polar cone cells. At 40-hr pupa the two primary pigment cells completely enwrap the cone cells. The contacts between the cone cells are reversed where the polar and the equatorial cone cells contact while the anterior and posterior cone cells are pushed away from each other. The total number of cells in the

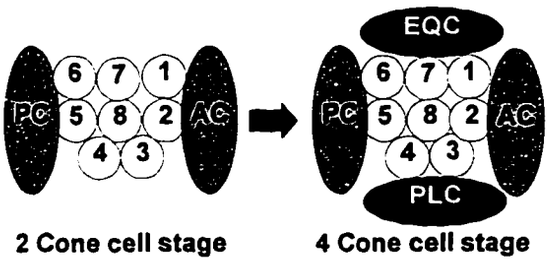
Figure 2. Pattern formation in the developing eye. (A) During the third larval instar development, pattern formation and cell differentiation take place in a sequential manner. The first cell to differentiate is R8 cell that induces R2 and R5 cells to differentiate then R3 and R4 forming five cell precluster. Soon after, additional cells R1, R6 and R7 join the precluster. (B) In the late third larval instar eye disc the anterior and posterior cone cells develop and form the two cone cell stage. This is followed by the development of the equatorial and polar cone cells establishing the four cone cell stage. (C) In the early pupal eye development, 20-hr after pupal formation, the cone cells spread over the apical tips of the R cells and two presumptive primary pigment cells differentiate around the cone cells. At 30-hr pupal eye, the two primary pigment cells form a collar around the cone cells. At 40-hr, two cells (2 asterisks) are positioned to compete for a horizontal secondary pigment cell and three other cells (3 asterisks) are positioned to become a single tertiary pigment cell. At 60-hr, the cells that fail to fill the position likely die and the cellular pattern of the lattice is completed. Anterior and posterior cone cells (medium gray), equatorial and polar cone cells (extra dark gray), primary pigment cells (light gray), three tertiaries (dark gray), and anterior to the right.

Figure 2. Pattern formation in the eye.

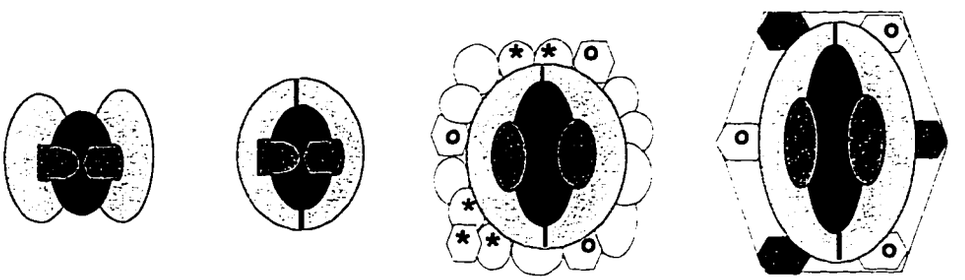
A: Mid-third larval instar stage



B: Late third larval instar stage



C: Early pupal stage (0-60hr)



surrounding lattice is reduced by programmed cell death (Cagan and Ready, 1989b; Wolff and Ready, 1991a). Studies on cell death have demonstrated that several cells compete for a single secondary or tertiary pigment cell fate, one cell succeeds to fill the position and establish appropriate contacts, and the others die. Finally, at about sixty hours of pupal formation the cellular pattern of the retina is completed [Fig. 2C].

Studies of mutations such as *Notch* have provided insights towards the understanding of the role of cell-cell interaction in cell fate determination and pattern formation in the eye. Cagan and Ready (1989a), found that mutations in *Notch* cause the cells to select inappropriate developmental pathway. Reducing *Notch* activity at the early stage of cellular development affects the photoreceptor and the cone cell fates, while reducing the activity at the late stage affects the primary and secondary pigment cell determination as well as the bristle differentiation in the eye. Their observations indicate the importance of the timing element in cell fate decisions. In another study, Tomlinson and Ready (1986), showed that the *sevenless* mutation causes the presumptive R7 cell to develop into a non-neural cone cell. Therefore, both timing and positional information are critical for the retinal cells to acquire their correct fates. Molecular and genetic studies have led to the formulation of models for the mechanisms by which cell fates are determined. One of these models is the Delta-Notch mediated signal transduction during *Drosophila* development. *Delta* and *Notch* are neurogenic genes that are required for correct cell fate choices of both neural and nonneural cells

(Muskavitch, 1994). These genes are also essential for wing vein formation (Lindsley and Zimm, 1992 cited from Muskavitch, 1994), and cone and pigment cell development in the retina (Cagan and Ready, 1989a). The best understood pathway for cell determination in the eye is the signal transduction cascade during the recruitment and specification of the R7 photoreceptor cell. Genetic mosaic analysis showed that the *sevenless* gene product (Sev) is required only in the R7 cell for its development (Campos-Ortega et al., 1979 cited from Venkatesh, 1993), while the Boss protein function is required only in the R8 cell for R7 cell differentiation (Reinke and Zipursky, 1988). Sev is a transmembrane receptor tyrosine kinase that is expressed in R7 cell and the presumptive cone cells (Hafen et al., 1987; Banerjee et al., 1987 and Tomlinson et al., 1987). The *boss* gene product (Boss) is a membrane protein that spans the surface of the R8 cell and is the ligand for the Sev receptor (Hart et al., 1990). Experiments by Kramer and co-workers (1991) indicated that Boss directly activates Sev. Although R7 cell precursor and cone cell precursors express Sev protein, only the cell that contacts the R8 cell develops through R7 pathway (Van Vactor et al., 1991). Mutations in *sev* or *boss* genes results in transformation of R7 precursor cell into a non-neuronal cone cell (Zipursky and Rubin, 1994). Identification of a group of genes called *Enhancers of sevenless* [*E(sev)*] that are involved in Sev signaling pathway led to further understanding of the signaling steps in the pathway that regulates the R7 development. The activation of Sev by Boss allows the initiation of the signal cascade. These interactions stimulate the conversion of inactive GDP-bound Ras1 to active GTP-bound Ras. Consequently, the activation of Ras1 results in the activation of a cascade

of protein kinases including Raf and Rolled kinases (Simon, 1994) [See Fig. 24]. Although the Sevenless protein is not required by any other cell than R7, Ras1 protein is required for all photoreceptors (Simon et al., 1991). In other studies, genetic interactions of the members of spitz group (*spitz, rhomboid, Star* and *pointed*) and the *Drosophila* homologue of the epidermal growth factor receptor (EGFr) indicate that they function in a common pathway (Freeman, 1994). The EGFr is required for both cell proliferation in the eye imaginal disc and photoreceptor cells determination (Xu and Rubin, 1993). Spitz is the ligand for EGFr during eye development. Spitz/EGFr interaction activates Ras1 dependent signal transduction pathway (Freeman, 1994). Freeman suggested that photoreceptor cells other than R7 cell use Ras1 signaling pathway activated by Spitz/EGFr interaction rather than Ras1 pathway activated by Boss/Sevenless interaction. This pathway may provide the basis for the recruitment of photoreceptors other than the R7 cell. On the other hand, *argos,* which is a repressor of cell determination, has opposite effects to EGFr on the eye. In *argos* mutants, mystery cells are transformed into extra photoreceptor, cone and pigment cells. Argos protein inhibits the activation of EGFr by Spitz either by directly acting as a Spitz competitor or by binding to an unknown receptor and indirectly inhibiting EGFr signaling (Schweitzer et al., 1995).

Although a number of studies have provided insights into the molecular mechanisms by which photoreceptor cell fates are determined, little is known about the mechanism involved in the nonneural cell fate determination. In this dissertation, I

have studied a rough-eyed mutation called *rugose* (*rg*) which affects the normal pattern formation of the eye through its effect on nonneural cell fate determination. In *rugose* mutants, neural cells (R cells) differentiate normally while nonneural cells (cone and pigment cells) are affected by the mutation. To understand some aspects of the nonneural cell fate determination, I have raised the following questions: What are the cellular defects caused by *rg* mutation? What is the precise nature of the role of *rg* in eye development? What is the molecular nature of *rg* gene? In which signaling pathway is *rg* involved? Does *rg* interact with other known genes and what are the effects of these interactions on *rg* gene function? and what is the *rg* specific role in the *Drosophila* pattern formation? In the following chapters, a phenotypic and genetic analyses of *rugose* gene are described. Developmental studies on *rg* mutant eyes have been carried out. Genetic interactions of *rugose* with genes involved in the EGFr pathway were also studied. This is followed by molecular analysis of the *rugose* locus in which I have made attempts at molecular cloning of the *rugose* gene. Together, these studies reveal that *rugose* is involved in cellular pattern formation in the developing eye through its effects in cone and pigment cell determination, and that *rugose* may function in EGF receptor mediated signal transduction pathway or a related pathway specific for nonneural cell determination.

CHAPTER II

PHENOTYPIC AND GENETIC ANALYSES OF THE *rugose* MUTATIONS

INTRODUCTION

One of the most important factors that have led to the progress in developmental biology is the use of genetic mutations. The study of mutant phenotypes and an understanding of the molecular basis of these phenotypes has allowed us to identify the roles played by the genes in cell development and differentiation. The *Drosophila* compound eye is readily amenable to isolation of mutants. The development of the *Drosophila* compound eye is an excellent system to study the role of cell-cell interactions in cell fate determination and pattern formation. In recent years genetic and molecular studies from several laboratories on the cellular pattern formation in the *Drosophila* retina have provided important insights towards our current understanding of the mechanisms involved in the development and function of the eye. The adult compound eye consists of nearly eight hundred unit-eyes called ommatidia, which are arranged in a regular symmetrical array. Each ommatidium contains 20 cells; eight retinular photoreceptor neurons (R cells), and in addition an invariant number of non-neural accessory cells. These accessory cells include four lens-secreting cone cells, six pigment cells and a mehanosensory bristle complex forming a sensory hair group (Ready et al.,

1976; Tomlinson, 1985; Tomlinson and Ready, 1987a,b; Cagan and Ready, 1989 and Wolff and Ready, 1991a,b). In each hexagonally shaped ommatidium the photoreceptor cells are organized into six outer cells (R1-R6) surrounding two central cells (R7 and R8). The plasma membrane of these cells is multiply folded in a serrate fashion forming a microvillar structure known as the rhabdomere which contains specific light sensitive photopigments. The rhabdomeres of the outer cells (R1-R6) extend the length of the retina, while those of the central cells (R7 and R8) are smaller and shorter. The R7 rhabdomere is positioned on top of the basal R8 rhabdomere. The photoreceptor cell axons break through the fenestrated basement membrane to the optic lobe. Axons from R1-R6 cells terminate in the first optic ganglion, the lamina, whereas axons from R7 and R8 cells terminate in the second optic ganglion, the medulla. The optic lobe development is dependent on the R cell axon innervation. The R cells axons project into the optic ganglia through the optic chiasma with precise connectivity patterns so as to represent the fly's visual field in the optic ganglia (Meyerowitz and Kankel, 1978).

Four cone cells reside on top of the R cells in each ommatidium and secrete the lens above the retina. Each of the four cone cells can be identified by its position. The anterior cone cell lies over R1-R3, the posterior cone cell overlie R4-R6, the equatorial cone cell is positioned on top of R1, R6 and R7 and the polar cone cell lies above R3 and R4 (Cagan and Ready, 1989 and Wolff and Ready, 1991). The eight R cells and the four cone cells are surrounded by the pigment cells which isolate them from the neighboring ommatidia. Two primary pigment cells surround the four cone cells.

Three secondary and one tertiary pigment cells together with the bristle complex form the outer hexagonal lattice. The secondary and tertiary pigment cell feet form the thin filamentous fenestrated basement membrane that support the retina. The cone cells contact each other at the fenestrated basement membrane level forming the cone cell feet (Cagan and Ready, 1989).

Pattern formation is initiated by neuronal differentiation during the third larval instar stage. A dorso-ventral groove called the morphogenetic furrow (MF), begins at the posterior edge of the eye imaginal disc, and progresses anteriorly leaving differentiated R cell-clusters behind. The morphogenetic furrow plays a critical role in eye development. It divides the eye into unpattern epithelium to the anterior and differentiating clusters to the posterior. The furrow moves from the posterior to the anterior by signals from the differentiating photoreceptor cells such as *hedgehog* (*hh*) (Heberlein and Moses, 1995). Treisman and Rubin (1995) showed that mutations that prevent the normal furrow progression such as *wingless* (*wg*) and *shaggy* (*sgg*) block cell differentiation. Once blocked the furrow does not reinitiate its progression or cell differentiation beyond the position of the block. However, no clear explanation has been established to link cell differentiation and the onset of pattern formation in the furrow.

Genetic mosaic studies by Ready and his colleagues (1976) showed that cell fate in the eye is not determined by cell lineage and, that, cells in each ommatidium are not derived from a single mother cell. Therefore, they suggested that cells are recruited and the cell types are determined according to their position within spatial

environment, and specific cell-cell contacts. Thus cell-cell interactions and positional information direct cell fate determination (Lawrence and Green, 1979).

Immunohistochemical studies employing monoclonal antibodies that recognize neuronal-specific antigens have demonstrated that the R cells differentiate in a specific sequence (Zipursky et al., 1984; Venkatesh et al., 1985). The model proposed by Tomlinson and Ready (1987) suggested that cellular pattern formation occurs by sequential inductive events. The nucleus of a cell rises from the basal to the apical surface of the eye imaginal disc immediately before differentiation. The first cell to differentiate is R8 cell, which induces the differentiation of R2 and R5 cells followed by R3 and R4 cells resulting in five-cell precluster. The precursors of the precluster cells are derived from a wave of mitosis anterior to the morphogenetic furrow. The R1 and R6 cells join the developing cluster and the last cell to differentiate is the R7 cell. These three R cells and all other future retinal cells are derived from a second mitotic wave immediately posterior to the furrow (Tomlinson, 1985). By the end of the larval life, the four cone cells differentiate. During the early pupal life, the cells surrounding the clusters develop into a precise pigment cell lattice and generate the array of sensory bristles (Tomlinson, 1985; Tomlinson and Ready, 1987 and Cagan and Ready, 1989). Undifferentiated cells are removed from the eye disc through programmed cell death (Wolff and Ready, 1991a,b).

In recent years, studies of several genes involved in eye pattern formation have highlighted the importance of cell-cell interactions in cell fate specification. In this chapter, I present data on the isolation and characterization of a number of *rugose*

alleles. These were used to determine morphological and cellular defects caused by the mutation. The phenotypic analysis of *rg* mutation presented here has uncovered the role of the *rugose* gene in cellular pattern formation in the developing compound eye.

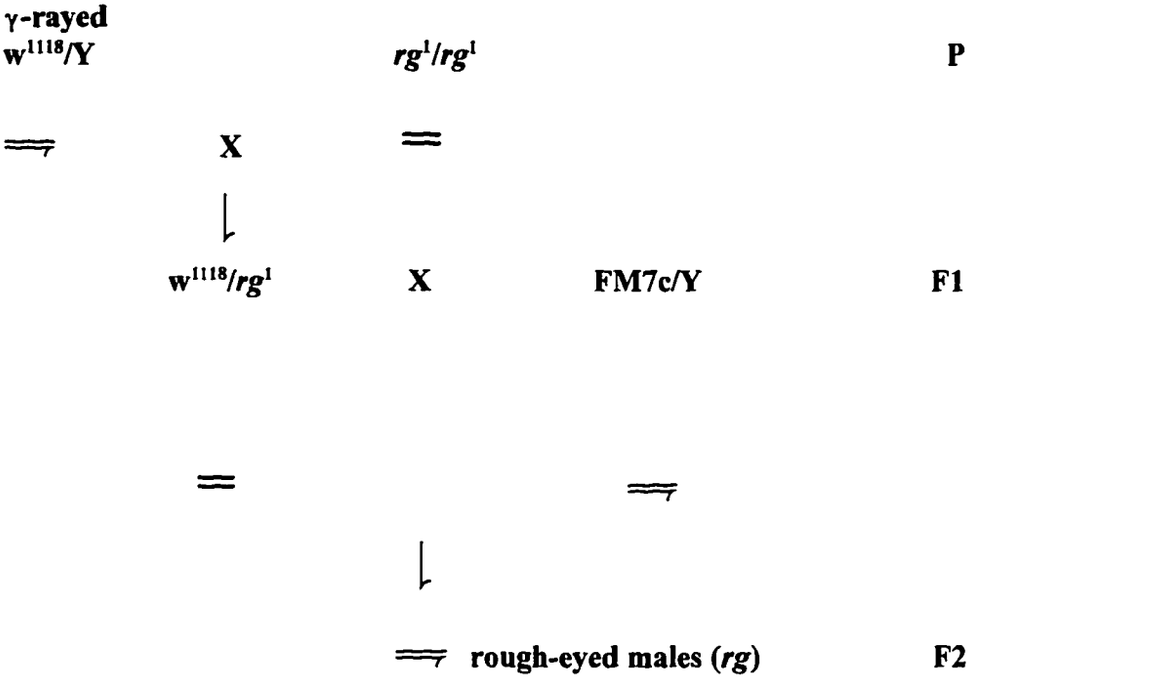
RESULTS

Isolation of New *rugose* Alleles

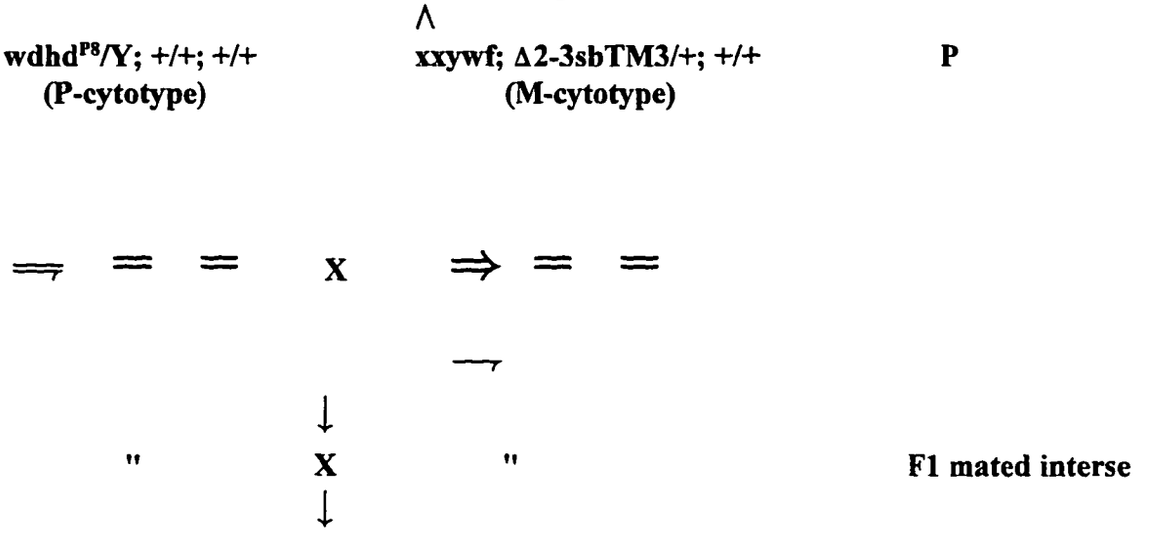
The original *rugose* mutation (rg^1) is a spontaneous recessive mutation on the X-chromosome. Twenty alleles of *rugose* were isolated and used in this study. Eleven γ-ray induced alleles of *rg* ($rg^{x\text{-}1}$ _ $rg^{x\text{-}11}$) were isolated by screening 16,100 flies. In addition, nine P-element-induced alleles of *rg* (rg^{p1}_ rg^{p9}) were isolated by screening 12,500 F2 flies in a hybrid dysgenic cross [Table 1]. Some of the isolated alleles such as $rg^{x\text{-}1}$, $rg^{x\text{-}3}$, $rg^{x\text{-}6}$, rg^{p3} and rg^{p5} behave like null alleles (complete loss of function mutations) by genetic criteria. The rough eye phenotype does not change when a mutant chromosome is placed over a chromosome carrying deficiency for the *rg* region. Homozygous *rg/rg* females, hemizygous males (*rg*/Y) and mutant females that carry a deficiency in one X-chromosome (*rg*/Df) showed similar eye phenotype. The remaining alleles appear to be hypomorphic alleles (partial loss of function mutations). The homozygous flies (*rg/rg*) showed a less severe eye phenotype than the mutant females that carry the *rg* mutation over a deficiency (*rg*/Df). In complementation tests, the isolated alleles failed to complement the original spontaneous rg^1 allele, indicating that these newly induced mutations are allelic to *rg*. The mutant stocks have variable

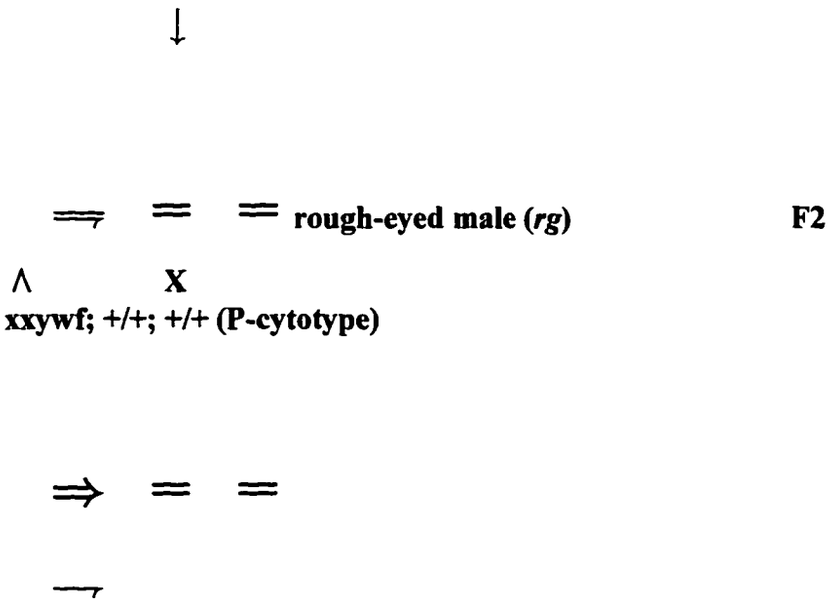
expressivity, where the degree of phenotypic expression of the gene varies from one individual to another. However, the *rg* mutant alleles exhibit 100% penetrance (ie. all individuals exhibit a mutant phenotype).

Isolation of γ-ray induced alleles of *rugose*



Isolation of P-element-induced alleles of *rg*





The isolated rough-eyed males were maintained and then tested by complementation with rg^1.

Isolation of Revertants

Hybrid dysgenic crosses were performed to remobilize the P element at the *rg* locus. This analysis was carried-out to test if the reversion leads to restoration of the phenotype and to determine whether the mutations were caused by P insertions or deletions. Two P-induced alleles rg^{p2} and rg^{p5} were selected for reversion analysis. rg^{p2} remobilization cross were screened and 33 revertant lines ($rvrg^{p2.1}$_ $rvrg^{p2.33}$) were isolated out of 2,100 F2 males scored, which represent 1.57% recovery. In rg^{p5} remobilization screen, 11 revertant lines ($rvrg^{p5.1}$_ $rvrg^{p5.11}$) were isolated out of 1000 F2 males representing 1.1% recovery [Table 2]. This high frequency of reversion suggests that the P-element-induced mutations were due to insertions rather than deletions at the gene locus. Some of these revertant lines showed male sterility as high as 30% in

rg^{p2} and 27% in rg^{p5} revertants. The established revertant lines are divided into complete and partial revertants based on their eye phenotypes. The partial reversion is consistent with imprecise excision of the inserted P-element.

Isolation of revertants

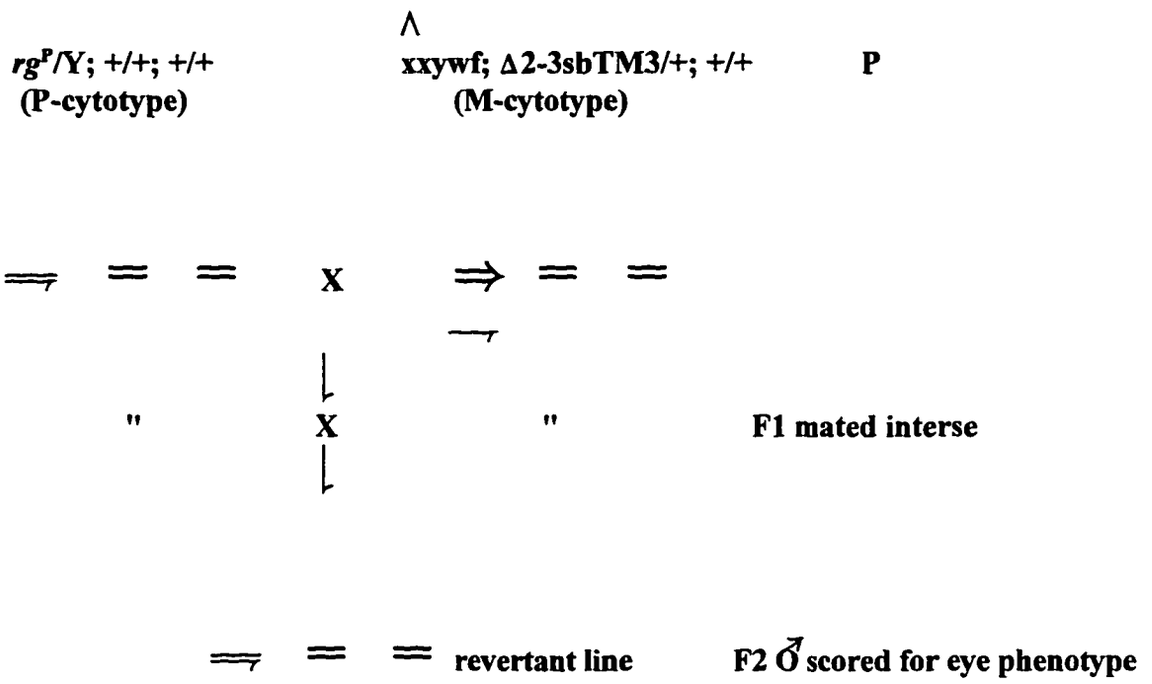


Table (1)

Isolation of *rugose* alleles by γ-ray mutagenesis and P-element induction

rugose alleles	*rugose* eye phenotype	Classification
rg^{p1}	+++	hypomorph
rg^{P2}	+++	hypomorph
rg^{P3}	++++	null
rg^{p5}	++++	null
rg^{p6}	+++	hypomorph
rg^{P9}	+++	hypomorph
rg^{X-1}	++++	null
rg^{X-2}	++	hypomorph
rg^{X-3}	++++	null
rg^{X-4}	++	hypomorph
rg^{X-5}	+++	hypomorph
rg^{X-6}	++++	null
rg^{X-7}	+++	hypomorph
rg^{X-8}	+++	hypomorph
rg^{X-9}	+++	hypomorph
rg^{X-10}	+++	hypomorph
rg^{X-11}	+++	hypomorph
rg^{1}	++	hypomorph

++++ = severe; +++ = moderate; ++ = mild

Table (2)

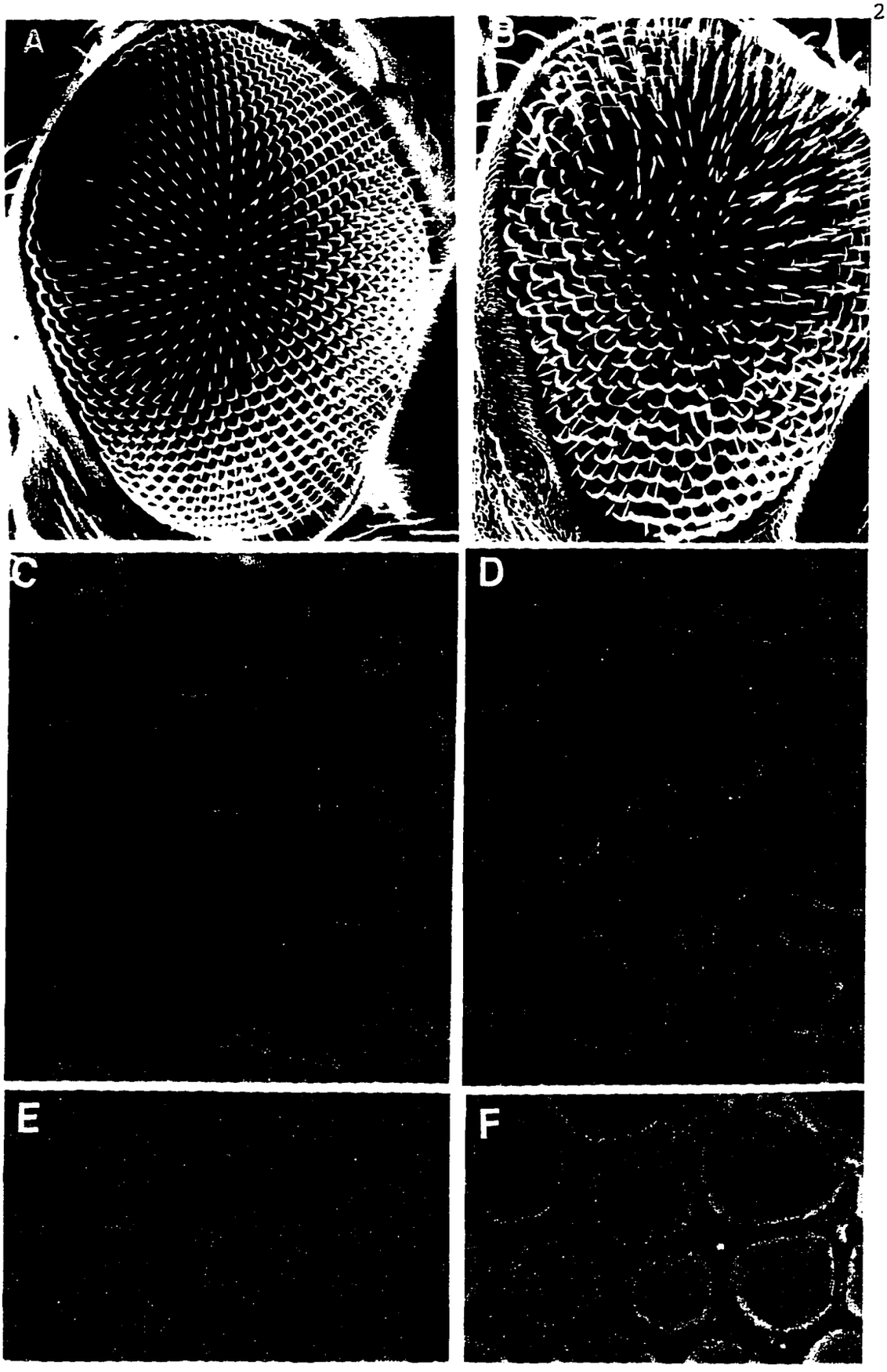
Reversion analysis

Parental Line	F2 males scored	Smooth eyed revertants	Sterile males	Revertant Lines	Frequency
rg^{p2}	2100	33	10	23	1.57%
rg^{p5}	1000	11	3	8	1.10%

Eye Phenotype of *rugose* Mutants

Mutations in the *rg* gene result in the abnormal rough eye pattern. The roughness of the eye could be classified from mild to severe depending on the particular allele. The structure and organization of the wild-type eye shows a smooth array of reiterated unit eyes or ommatidia [Fig. 3A,C,E]. On the other hand, *rg* mutant eyes exhibit an irregular array, multiple mechanosensory bristles, defective lattice and unequal sized ommatidia [Fig. 3B,D,F]. Occasionally, *rg* mutants show fusion of two ommatidia and patches of solely pigment cells in many positions [Fig. 4A,B]. In some ommatidia abnormal number of photoreceptor cells (R cells) was observed. In these ommatidia the number of R cells is fewer than the normal seven cells that are seen in a given tangential section of the wild-type ommatidium. In tangential sections of the *rugose* mutant eye, abnormal packaging of the ommatidia was observed. This leads to irregular arrangement of the photoreceptors in each ommatidium. Some R cells appear larger than usual pushing the other R cells into a smaller space [Fig. 4C,D].

Figure 3: Morphology and anatomy of the wild-type and *rg* mutant compound eyes. Scanning electron micrographs of (A) Wild-type showing smooth eye with regular array (200X) and (B) rg^{x-6} compound eye showing rough eye with irregular array (200X). Tangential sections of (C) Wild-type showing regular arrangement of R cells in each ommatidium and (D) rg^{x-6} showing abnormal arrangement of ommatidia and R cells, and aberrant pigment cell lattice. Outer tangential sections showing the matrix of secondary and tertiary pigment cells in (E) Wild-type showing regular hexagonally shaped matrix that surrounds equally sized lenses and (F) rg^{x-6} showing irregular shape of the matrix and different sizes of the lenses (1000X). Anterior to the right.



These cells do not appear to be aligned in any particular pattern. The irregular arrangement of the R cells gives the eye a rough external surface. In some alleles the fusion of several ommatidia were observed as in *rg*^{p5} [Fig. 4E,F]. In another allele of *rugose*, *rg*^{p3}, vacant spaces were found in between ommatidia [Fig. 4G,H]. Flies grown at 17°C showed mildly rough eyes compared to flies grown at 24°C or 29°C [Table 3] and [Fig. 5A-D].

Normal Cell Division And Cell Death In *rugose* Mutants

In the developing wild-type third larval instar eye discs two bands of mitosis can be seen by bromodeoxyuridine (BrdU) labeling. BrdU labels cells undergoing DNA synthesis in mitotic divisions. The first mitotic wave is ahead of the morphogenetic furrow (MF) and the second mitotic wave is seen posterior to the furrow. The first mitotic division generates precursors for the formation of the five R cells of the precluster, R2-R5 and R8 (Ready et al., 1976). The second mitotic division results in the formation of the remaining retinal cells R1, R6, R7, the cone cells, and pigment cells (Cagan and Ready, 1989; and Wolff and Ready, 1991a,b). The two BrdU labeled bands are seen in the *rg* mutant eye discs similar to the wild type [Fig. 7A,B]. These results were confirmed by using antibodies to Cyclin (PCNA) which labels cells undergoing G1 and G2 phases in mitotic division. These studies also revealed normal mitotic pattern in *rg* mutants.

Figure 4: Phenotype of various mutant alleles of *rg*. (A) A scanning electron micrograph (SEM), and (B) A tangential section of $rg^{x\text{-}10}$ compound eye shows fusion of two ommatidia and defective lattice. (C) SEM, and (D) a tangential section of $rg^{x\text{-}3}$ compound eye shows some large size R cells (asterisks). (E) SEM, and (F) a tangential section of rg^{p5} compound eye shows fusion of three ommatidia. (G) SEM, and (H) a tangential section of rg^{p3} compound eye shows some vacant spaces between ommatidia that reveals more disrupted lattice. SEM (200X) and tangential sections (1000X). Anterior to the right.

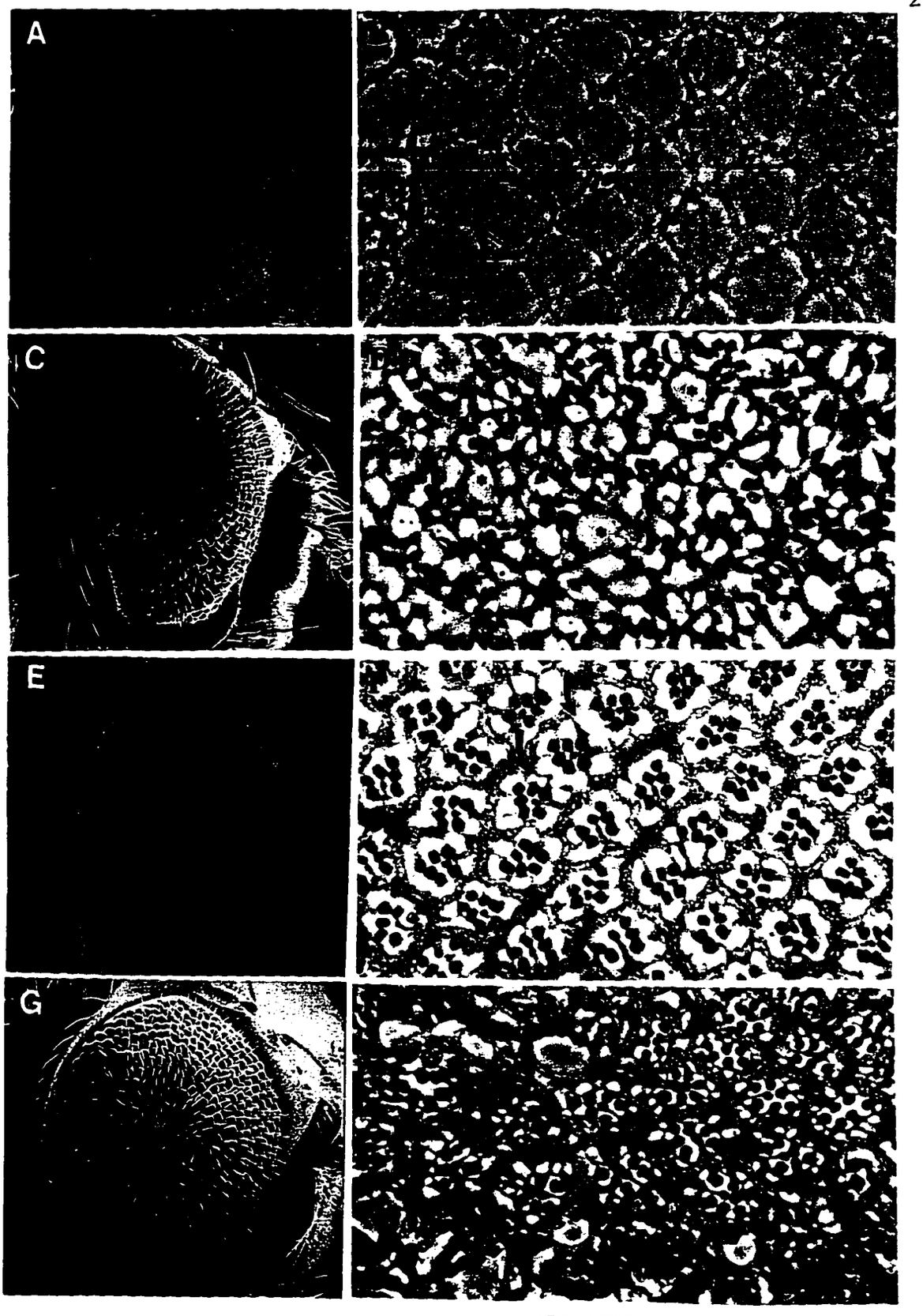


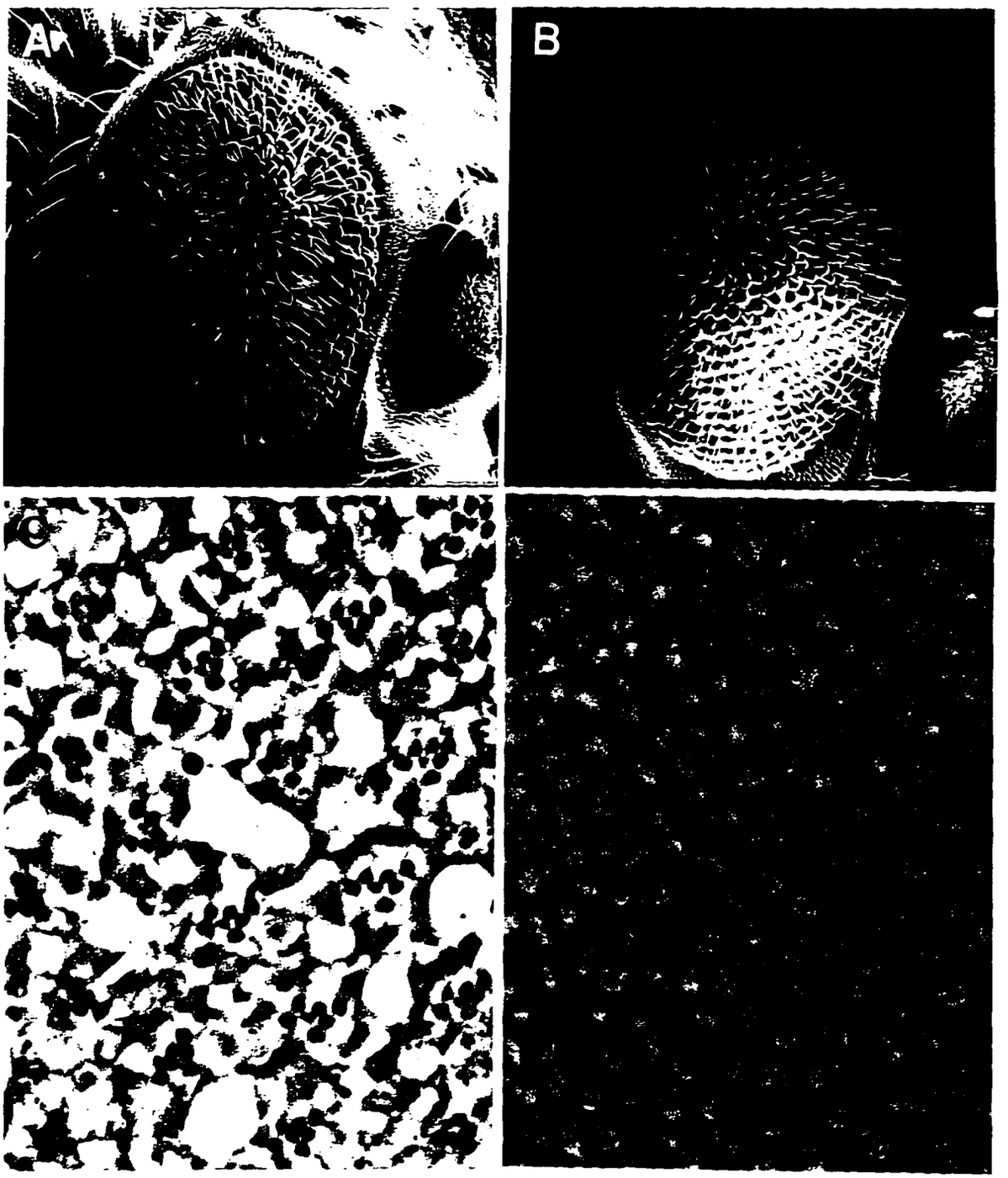
Table (3)

Comparative studies of the *rugose* eye phenotypes

rugose alleles	Classification	*rugose* eye phenotype at 17°C	*rugose* eye phenotype at 24°C	*rugose* eye phenotype at 29°C
rg^{P1}	hypomorph	+++	+++	+++
rg^{P2}	hypomorph	+++	+++	++++
rg^{P3}	null	++	++++	++++
rg^{P5}	null	++	++++	++++
rg^{P6}	hypomorph	+++	+++	++++
rg^{P9}	hypomorph	+++	+++	++++
rg^{X-1}	null	+++	++++	++++
rg^{X-2}	hypomorph	++	++	+++
rg^{X-3}	null	++	++++	++++
rg^{X-4}	hypomorph	++	++	+++
rg^{X-5}	hypomorph	++	+++	+++
rg^{X-6}	null	++	++++	++++
rg^{X-7}	hypomorph	++	+++	++++
rg^{X-8}	hypomorph	++	+++	++++
rg^{X-9}	hypomorph	++	+++	++++
rg^{X-10}	hypomorph	++	+++	++++
rg^{X-11}	hypomorph	++	+++	++++
rg^{1}	hypomorph	++	++	+++

++++ = severe; +++ = moderate; ++ = mild

Figure 5: Phenotypes of the *rg* mutants compound eyes grown at different temperatures. (A) A scanning electron micrograph of the compound eye of rg^{x-3} grown at 29°C showing a severe rough eye phenotype (200X); and (B) the compound eye of rg^{x-3} grown at 17°C showing a mild phenotype (200X). Tangential sections through (C) rg^{x-3} eye (29°C) showing disrupted lattice and abnormal packaging of the ommatidia, and (D) rg^{x-3} eye (17°C) showing fewer defects in the lattice. 1000X. Anterior to the right.

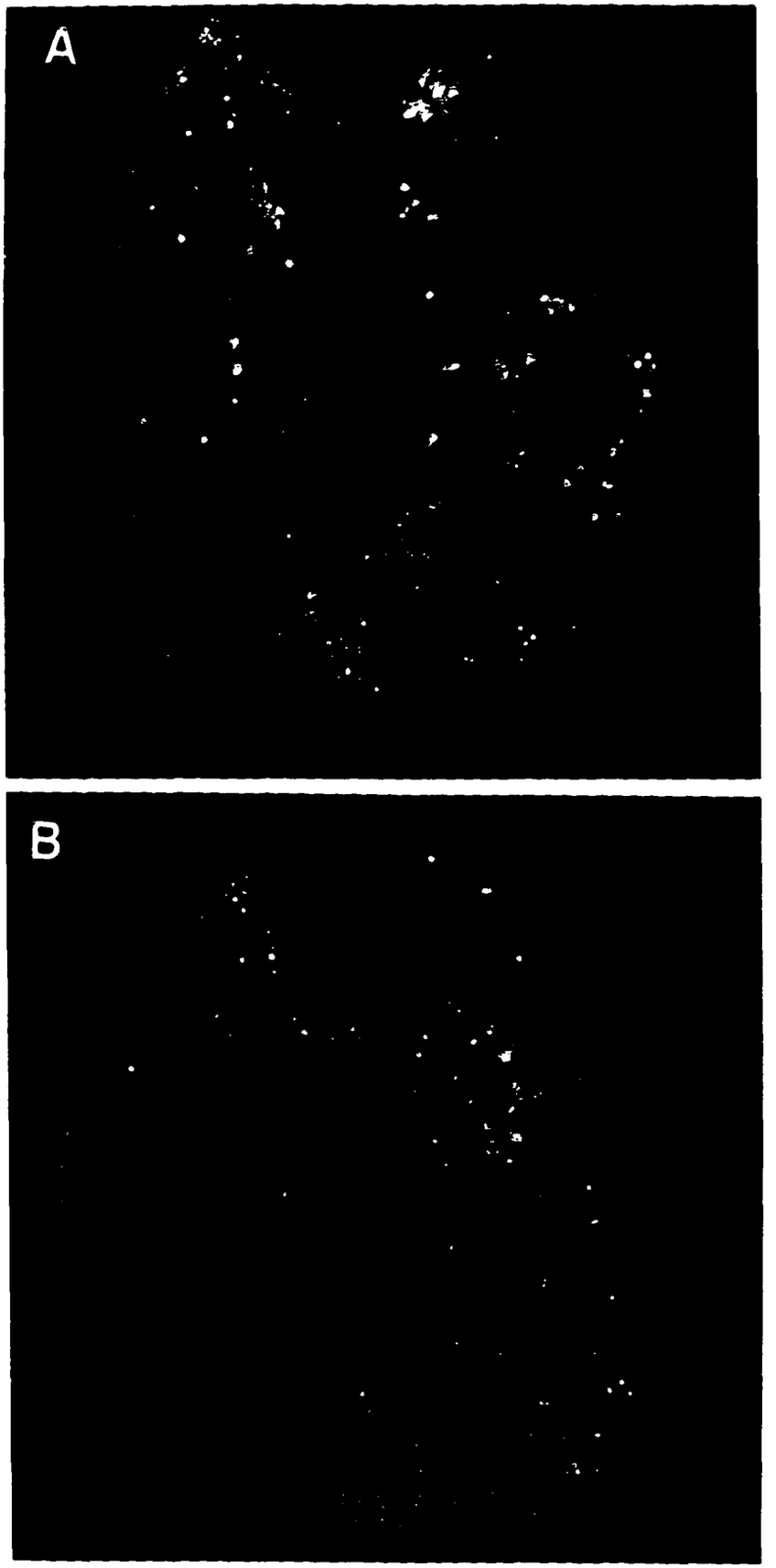


The precise cellular pattern of the compound eye is achieved by the elimination of surplus cells through programmed cell death. The first phase of cell death occurs at the third instar and the second phase is between 35 and 50hr of pupal formation (Wolff and Ready, 1991a). This cell death is easily visualized by staining with acridine orange. To test if the cell death patterns in *rg* mutants were abnormal, I stained developing *rg* mutant eyes with acridine orange. In both of wild-type and *rg* mutant eye discs, acridine orange staining showed two bands of cell death one anterior and another posterior to the MF [Fig. 6A,B]. Cell death in late pupal eyes was also tested and no differences between the wild-type and the *rg* mutant eyes were observed.

Photoreceptor Cell Differentiation is Not Affected in *rugose* Mutant Eyes

In the developing eye the differentiation of the R cells follows a specific sequence. The first cell to differentiate is the R8 cell which induces a pair of R cells, R2 and R5 to differentiate. This is followed by the differentiation of R3 and R4. Subsequently R1 and R6 differentiate and, the last cell to differentiate is the R7 cell. This pattern of differentiation can be followed by using neuronal-specific antibodies such as MAb22C10 (Zipursky et al., 1984 and Venkatesh et al., 1985). Although abnormal number of R cells were observed in some *rugose* mutant ommatidia (20% of mutant ommatidia), normal specification and differentiation of R cells were detected as evidenced by normal MAb22C10 staining. In addition to MAb22C10 a variety of R cell specific molecular markers are available. I have used some of these to follow the

Figure 6: Cell death in the third larval instar eye discs. (A) Wild-type and (B) *rg* mutant eye discs showing acridine orange-stained fragments of dying cells. There is no significant difference between the wild-type and the *rg* mutant cell death as they show comparatively equal levels of dying cells. 400X. Anterior to the left.



differentiation of the R cells in *rugose* mutants. The photoreceptor R8 cell is the first cell to differentiate and Kramer et al. (1991) have shown that the expression of Boss protein (*bride of sevenless*) is specific to R8 cells. I have used anti-Boss antibodies to follow the differentiation of R8 cells in *rg* mutants. Normal anti-boss antibody staining was observed in third larval instar *rg* mutant eye-imaginal discs. Posteriorly behind the morphogenetic furrow, Boss expression can be seen in single cells arranged in a regular pattern [Fig. 7C,D]. Mlodzik et al. (1990a), and Baker et al. (1990) suggested that *Scabrous* (*Sca*) function is required only in R8 cells. They detected Scabrous expression ahead of the furrow before R8 cell differentiation, as well as, its expression in specifically R8 cells shortly behind the furrow. They concluded that Scabrous protein plays a role in the normal spacing of the R8 cells in the developing eye. In *rugose* mutant eye discs, normal Sca expression pattern in two bands of cells (anterior and posterior to the furrow), that express specific antigen against *Sca* antibody, was observed. The differentiation of other R cells was studied by using P-LacZ enhancer trap lines which express β-galactosidase in subsets of R cells (Bier et al., 1989). The *sevenup* (*svp*) gene function is required in R3, R4, R1 and R6 cells for normal ommatidial pattern formation (Mlodzik et al., 1990b). The *svp* enhancer trap line shown β-gal expression initially in R3 and R4 a few rows posterior to the MF. This is followed by β-gal expression in R1 and R6 giving rise to a four cell specific expression pattern. In rg^{x-6} background normal labeling in four R cells (R3, R4, R1 and R6) was detected in larval eye discs that carried *svp* enhancer trap on the second chromosome [Fig. 7E]. The *rhomboid* (*rho*) gene function is required and expressed in R8, R2 and

R5 cells (Freeman et al., 1992). In *rg*^{x6}/*rho* heterozygous male larval eye discs, three R cells (R8, R2 and R5) were normally labeled [Fig. 7F]. In *rg* mutants the rho-enhancer trap expression in R8, R2 and R5 was unaffected suggesting normal differentiation of these cells. Moreover, transmission electron microscope study showed the normal differentiation of eight photoreceptor cells in *rugose* mutant late third larval instar eye discs (see chapter 3 Fig. 13). Taken together, I conclude that in *rugose* mutants the photoreceptor cells are specified normally and that mutations at the *rugose* locus do not affect the R cells differentiation.

Abnormal Cone Cell Differentiation and Pigment Cell Lattice Formation in *rugose* Mutants

A comparative study of the pupal development were carried-out to look for defects in cone and pigment cells. Both of the wild-type and selected *rg* alleles at the white pre-pupal stage were collected and allowed to develop until the end of the pupal stage when hatching occurs. Thus, the time required for pupal development was determined for each allele. The percentage of pupal development 50-hr after pupariation was calculated. The results indicated that in the *rg*^{p5} allele development occurs at a rate slower than the wild-type (CS) while in other *rugose* alleles the rate of development was similar to the wild type [Table 4].

Figure 7: Cell division and R cell differentiation in the third larval instar eye discs. BrdU labels two bands of cells undergoing mitotic division in both (A) Wild-type and (B) *rg* mutant larval eye-imaginal discs (400X). The first mitotic wave anterior to the morphogenetic furrow (MF), appears less organized than the second mitotic wave posterior to the furrow. Boss antibody staining in R8 cells in both (C) Wild-type and (D) rg^{x-6} larval eye discs, illustrates regular pattern of R8 cell distribution in the developing preclusters (1000X). Enhancer-trap-lines label specific R cells. (E) Staining of *seven-up* (*svp*) enhancer-trap-line is detected by the expression of β-galactosidase in R3, R4, R1 and R6 in svp/rg^{x-6} larval eye disc (1000X). (F) Staining of *rhomboid* (*rho*) enhancer-trap-line is detected in R8, R2 and R5 in rho/rg^{x-6} larval eye disc (1000X). Anterior to the left.

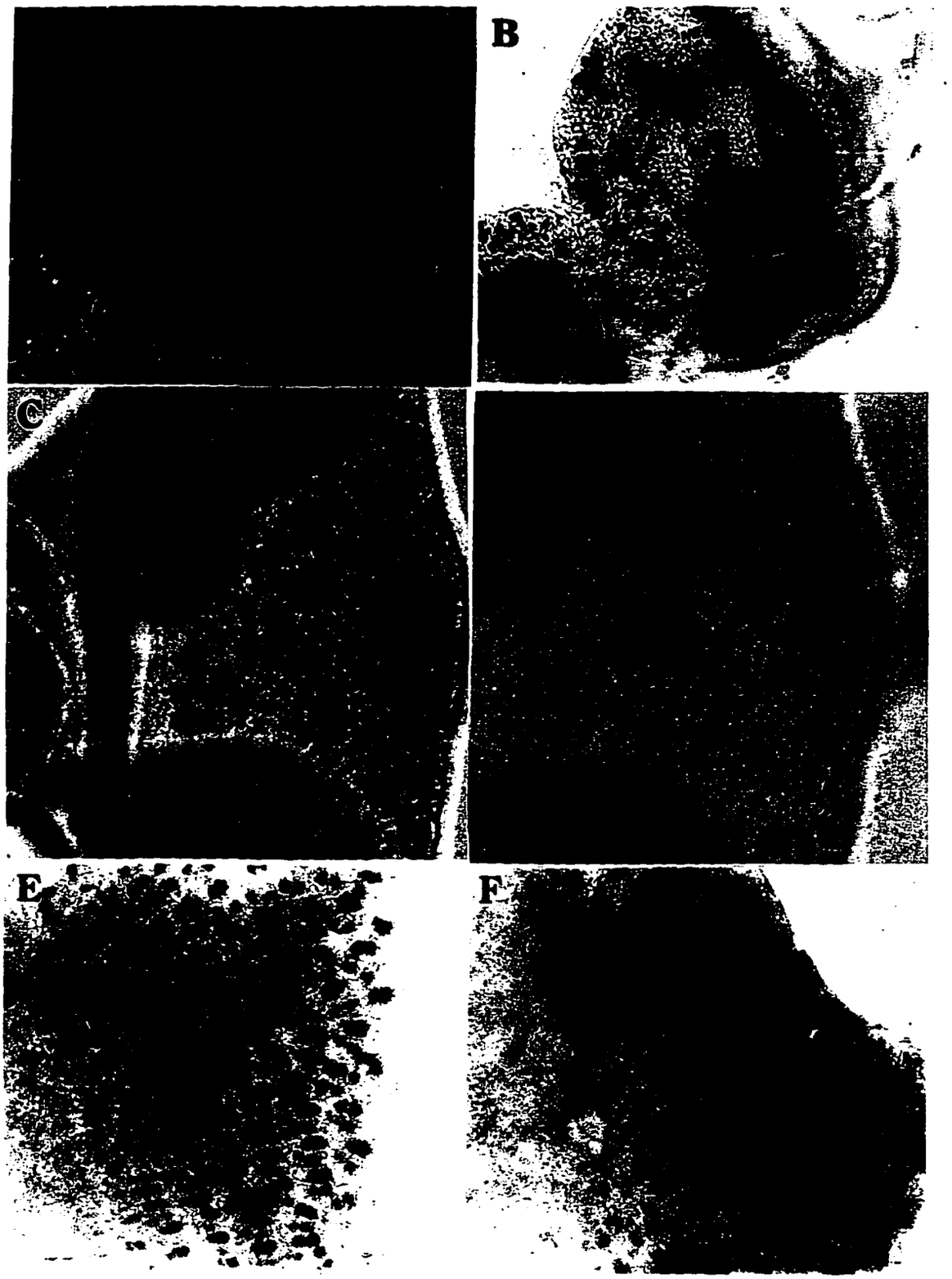


Table (4)

A comparative study of pupal development

Flies tested	Time required for 100% pupal development	% of Development 50-hr after pupal formation	Number of flies tested
CS	115 hr.	43.4 %	103
rg^{x-6}	116 hr.	43.1 %	127
rg^{x-10}	120 hr.	41.6 %	72
rg^{p2}	117 hr.	42.7 %	77
rg^{p5*}	125 hr.	39 %	73

* *rg*p5 pupal development is slower than Cs and other *rg* alleles.

The differentiating cone and pigment cells can be visualized by staining eyes at early pupal stages with cobalt sulfide which highlights cell membranes (Cagan and Ready, 1989b). Cobalt sulfide staining of *rg* mutant 50hr pupal eyes showed abnormal number of cone cells and pigment cell lattice formation. In wild-type 50hr pupal eyes, four cone cells surrounded by two primary pigment cells and normal lattice were observed [Fig. 8A]. Hypomorphic alleles of *rg* such as rg^{x-10} and rg^{p2} showed mostly 3 and 4 cone cells surrounded by two primary pigment cells [Fig. 8B,E]. Some cone cells failed to adopt their normal polarity [Fig. 8C,G]. Null alleles showed more severe defects. Two or fewer cone cells and abnormal development of pigment cell lattice was observed. The orientation of individual clusters was completely lost [Fig. 8C,D,G]. In the null allele rg^{x-6} the majority of ommatidia have two cone cells. The arrangement of ommatidia as well as the cone cells was abnormal in which it was difficult to indicate

the precise position of each cell [Fig. 8C]. The external lattice was highly disturbed showing extra secondary and tertiary pigment cells. This disrupted pattern resulted in abnormal bristles positions [Fig. 9A]. In the most severe case, in the rg^{x-3} allele, majority of the ommatidia completely lack cone cells with severe defects in the lattice [Fig. 9B]. No differences were observed between pupal eyes grown at higher or lower temperatures than 24°C. In all cases *rugose* mutant eyes showed fewer cone cells, normal primary pigment cells and a defective pigment cell lattice. No clusters with more than four cone cells was ever seen. Restoration of the cone cell number and the normal arrangement of the lattice was observed in the revertant eye discs [Fig. 8E-H]. Estimation of cone cell number in different alleles of *rg* suggested that cone cell number is sensitive to the *rg* gene dosage. In the null alleles 60% of ommatidia contain 0-2 cone cells [Fig. 10]. These results suggest that mutations in the *rg* gene lead to abnormal number and position of cone cells, and a disrupted pigment cell lattice. Thus, *rg* gene product is required for normal cellular pattern formation in the *Drosophila* compound eye.

Figure 8: Cobalt sulfide staining illustrates abnormal number of cone cells and defects in the pigment cell lattice in various *rg* mutant 50hr pupal eyes. (A) Wild-type shows a complement of four cone cells (c) in each ommatidium, surrounded by two primary pigment cells (Pp) and normal lattice. (B) $rg^{x\text{-}10}$ shows three and four cone cells/ommatidium. (C) $rg^{x\text{-}6}$ shows one or two cone cells/ommatidium, disrupted lattice and abnormal bristles positions. (D) $rg^{x\text{-}3}$ shows fewer ommatidia in the retina. the existing ommatidia have only one or no cone cells (arrows). (E) rg^{p2} shows two and three cone cells/ommatidium. (F) revertant rg^{p2} shows the restoration of cone cells back to four cone cells/ommatidium. (G) rg^{p5} shows one or two cone cells/ommatidium and multiple pigment cells in the surrounding lattice. (H) revertant rg^{p5} ($rvrg^{P5}$) showing mostly four cone cells/ommatidium and multiple pigment cells which reveals incomplete reversion. 1000X

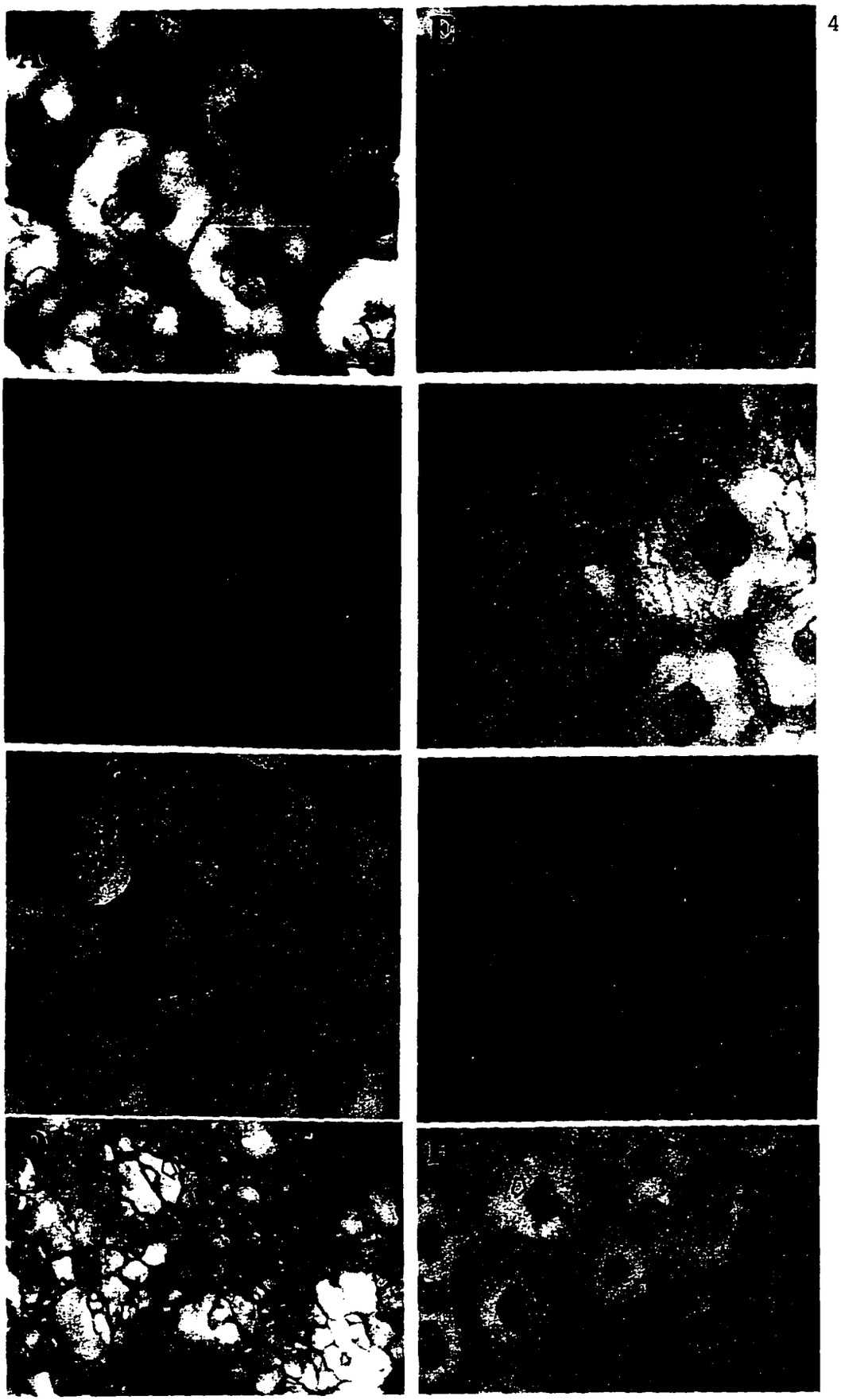
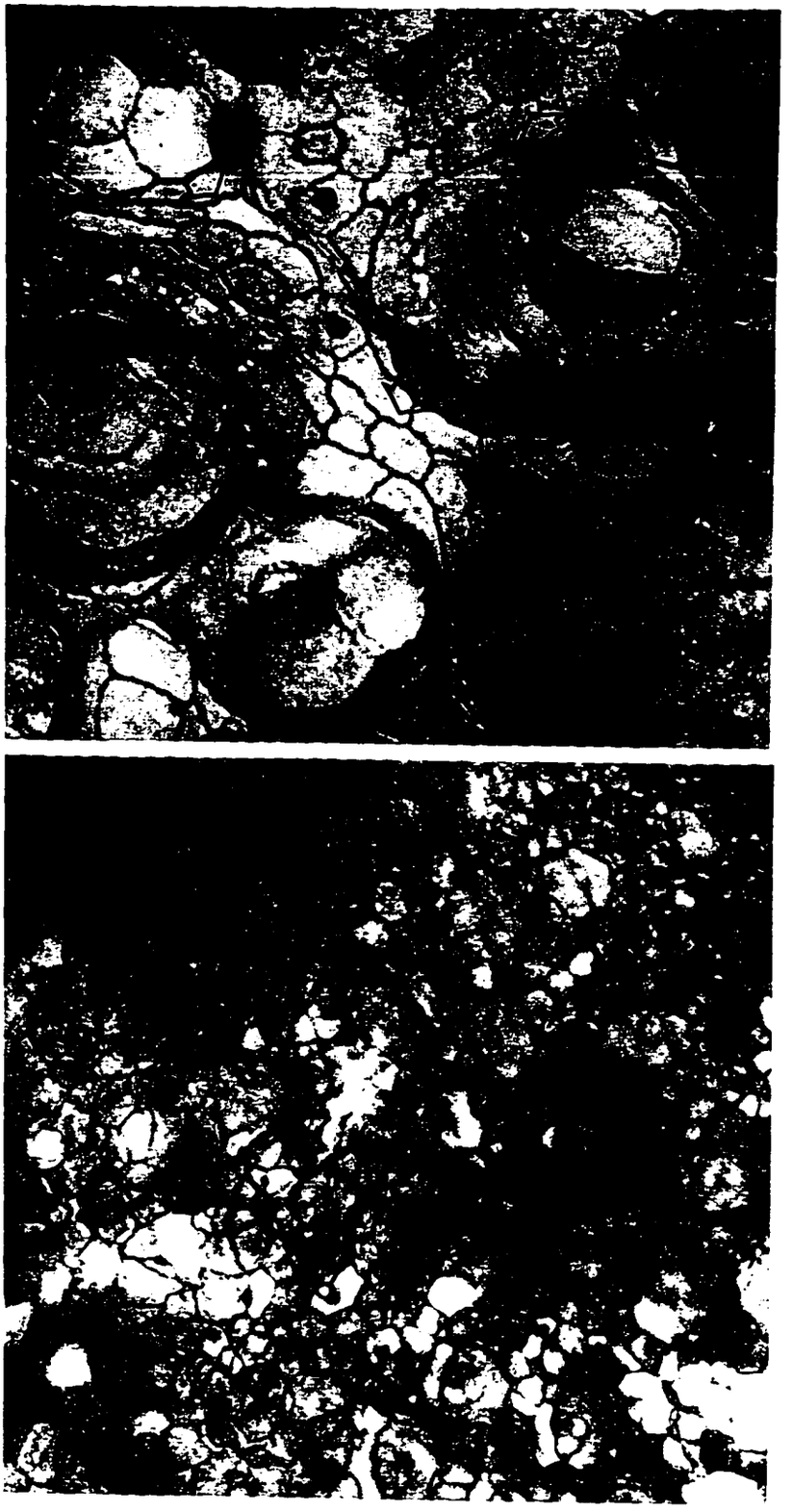


Figure 9: Cobalt sulfide staining reveals abnormal number and distribution of pigment cells in the lattice. (A) rg^{x-6} 50hr pupal eye shows multiple secondary and tertiary pigment cells between the ommatidia. (B) rg^{x-3} 50hr pupal eye shows accumulation of pigment cells in some areas in the retina (astrisks) that lack ommatidia. 1000X



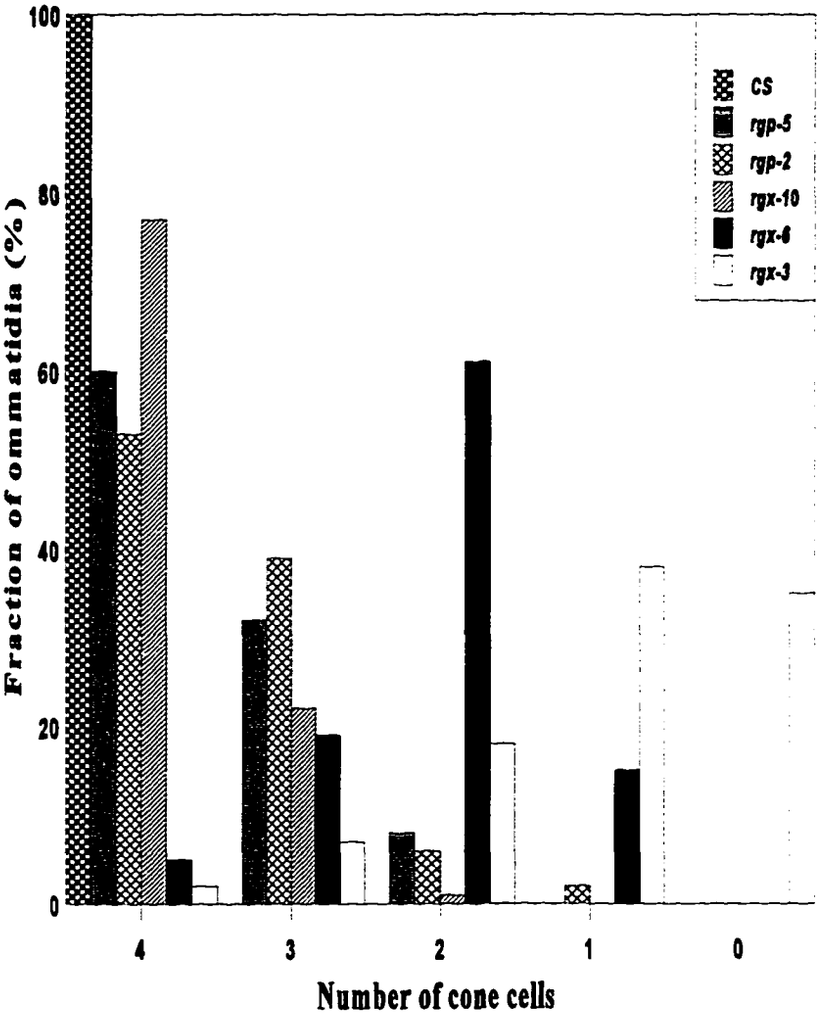
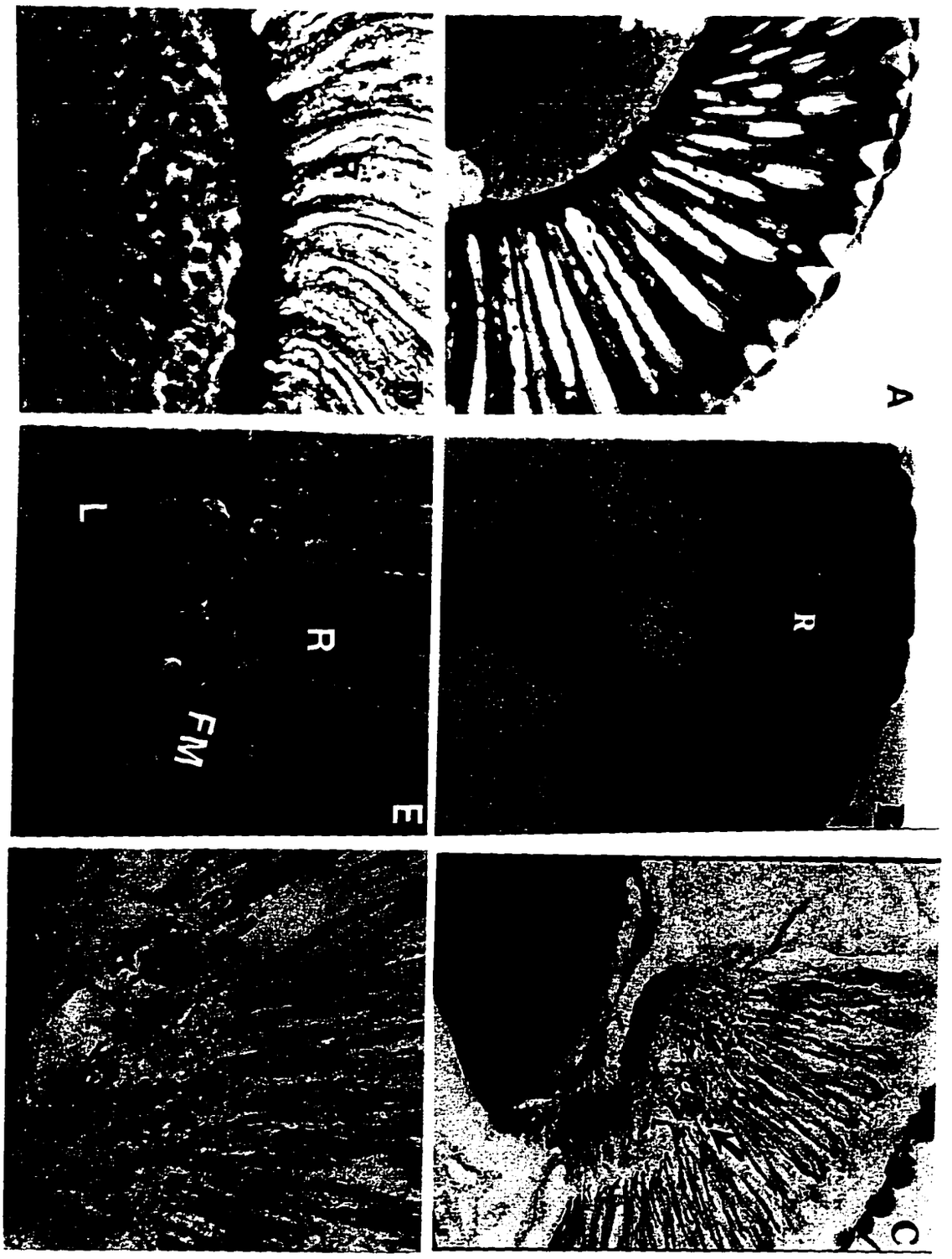


Figure 10: A histogram shows the distribution of cone cell numbers in various mutant alleles of *rg*. Null alleles (such as rg^{x6} and rg^{x3}) show mostly 0-2 cone cells/ ommatidium, while hypomorphic alleles (such as rg^{x10}) show mostly normal 4 cone cells/ ommatidium. Cone cell number is sensitive to the *rugose* gene dosage.

Mutation In *rugose* Affects The Fenestrated Basement Membrane Structure

The fenestrated basement membrane is a new retinal floor which is formed above and parallel to the original basement membrane. Cells release their anchorage to the original basement membrane and migrate to the apical surface of the eye disc, then they extend their feet posteriorly to the new membrane. Cagan and Ready (1989) have shown that the feet of the secondary and tertiary pigment cells flatten and widen developing into a filamentous fenestrated membrane (FM), which forms the floor of the retina. The cone cell feet enlarge and touch each other at the FM level. Axons derived from the R cells and the sensory bristles penetrate the FM into the optic ganglia. Transverse sections of the heads of *rg* mutant flies showed abnormal structure of the FM. In *rg* mutants a number of pores were observed in the fenestrated membrane leading to a disrupted basement boundary. Therefore, some retinular cells have no support at the base of the retina. Consequently, some of the R cells fall beneath the deformed floor of the retina and reach the lamina layer in the optic lobe [Fig. 11A-F]. Both toluidine blue staining and silver staining showed that the laminal cell body layer is thicker than that in the wild-type eye. This layer contained small darkly stained R cell rhabdomeres [Fig. 11E,F]. No defects in the axonal patterning at the medulla or the lobula levels were observed.

Figure 11: Mutation in *rg* affects the fenestrated basement membrane structure. Transverse sections of the wild-type (A&D) and *rg* mutant (B, C, E &F) heads. Note the disrupted fenestrated membrane in the *rg* mutant eyes. Collapsed photoreceptor cells are seen in the lamina region of the optic lobe. A,B,D and E were stained with toluidine blue stain. C and F were stained with silver stain. A,B and C (400X); D, E and F (1000X). R=retina, L=lamina, M=medulla, FM=fenestrated basement membrane.



Wing Phenotypes of *rugose* Mutants

In addition to the eye phenotype, *rugose* mutants also exhibit wing phenotypes which are temperature sensitive. In wild-type wings, the normal wing includes five wing veins, the marginal vein L1 and four longitudinal veins L2, L3, L4 and L5. In addition, two cross veins, the anterior cross vein in between L3 and L4, and the posterior cross vein between L4 and L5 [Fig. 12A]. Defective wings were found in *rg* mutants showing variable expressivity and low penetrance, where the defects were observed either in one or both wings of few individuals. The penetrance (ie. the fraction of flies showing the mutant phenotype) varied among different alleles of *rg* and was from 0 to 37.8% of the screened flies [Table 5]. Four different wing phenotypes were observed in *rugose* mutant flies, shrunken wings, curved wings, wings with incomplete venation (only at 17°C and 24°C), and wings with extra veins (only in 29°C) [Tables 6, 7, and 8]. Curved wings with multiple bristles were found (0-4.9%). The wings were bent upward and many dark bristles were seen [Fig. 12B]. Shrunken wings were observed in most of the *rg* alleles with penetrance ranging between 0-18.5% in which the wing looks damaged and folded [Fig. 12C]. Mutations in *rugose* also showed defective wing venation (0-36%), which is also temperature sensitive [Table 9]. Mutant flies grown at 17°C and 24°C showed incomplete L5 wing veins or incomplete cross veins. The longitudinal vein L5 terminated prematurely and did not reach the wing margin [Fig. 12D]. In some *rg* mutant alleles, short posterior cross veins were observed [Fig. 12H]. In contrast, *rg* mutants grown at 29°C showed extra veins. The longitudinal

vein L5 was completed and extra cross veins were formed. In rg^{x-1} two anterior or two posterior cross veins were seen, while in rg^{p3} two anterior and two posterior cross veins were observed [Fig. 12E,F,G]. In addition to the multiple cross veins, other alleles showed the development of short veins in unexpected positions. In rg^{x-3} and rg^{x-6} wings cross veins were seen in between L1 and L2, while rg^{x-11} wing showed the development of a cross vein between L2 and L3 [Fig. 12I,L]. In rg^{x-1} the cross vein between L4 and L5 formed one or two delta shapes [Fig. 12J,K]. Normal wing phenotype was found in rg^{p2} and rg^{p5} revertant lines. These results indicate that *rg* function is also required for normal differentiation of cells forming the wing veins. This wing phenotype is very interesting because other mutations that interact with *rugose* also show defective wing venation phenotype (see chapter 4). Both wild-type and *deadhead* flies were used as controls and showed normal wing development.

rugose Mutants Exhibit a Semi-lethal Phenotype

A number of *rugose* alleles exhibited a semi-lethal phenotype compared to the wild-type. The extent of lethality varied among different alleles of *rg* depending on the strength of the mutations. In the null alleles rg^{x-6} and rg^{x-3}, 40-60% of the embryos failed to hatch. Salz (1992), and Lindsley and Zimm (1992) have also reported semi-lethal phenotype of *rugose*. This suggests that mutations in the *rg* locus may cause defects in the early embryonic stages. Thus, in addition to the role of *rg* in the eye and wing development, *rg* may play a general (essential) role in *Drosophila* development.

Figure 12: Wing phenotypes of various *rg* mutant alleles. (A) A wild-type wing shows normal longitudinal veins L1-L5, an anterior cross vein (Acv) between L3 and L4, and a posterior cross vein (Pcv) between L4 and L5. B-L show samples of the defective wing phenotypes seen in the *rg* mutants. (B) A curved wing with multiple bristles (rg^{p5}). (C) A shrunken wing (rg^{x-1}). (D) Incomplete L5 vein (rg^{p3}). (E) Two anterior cross veins (rg^{x-4}). (F) Two posterior cross veins (rg^{p3}). (G) Two anterior and two posterior cross veins (rg^{p3}). (H) Incomplete posterior cross vein (rg^{x-1}). (I) Abnormal position of a cross vein seen between the marginal longitudinal vein L1 and L2 (rg^{x-3}). (J) A posterior cross vein forming a delta shape (rg^{x-1}). (K) A posterior cross vein forming two delta shapes (rg^{x-1}). (L) A cross vein located between L2 and L3 (rg^{x-11}). Anterior to the left.

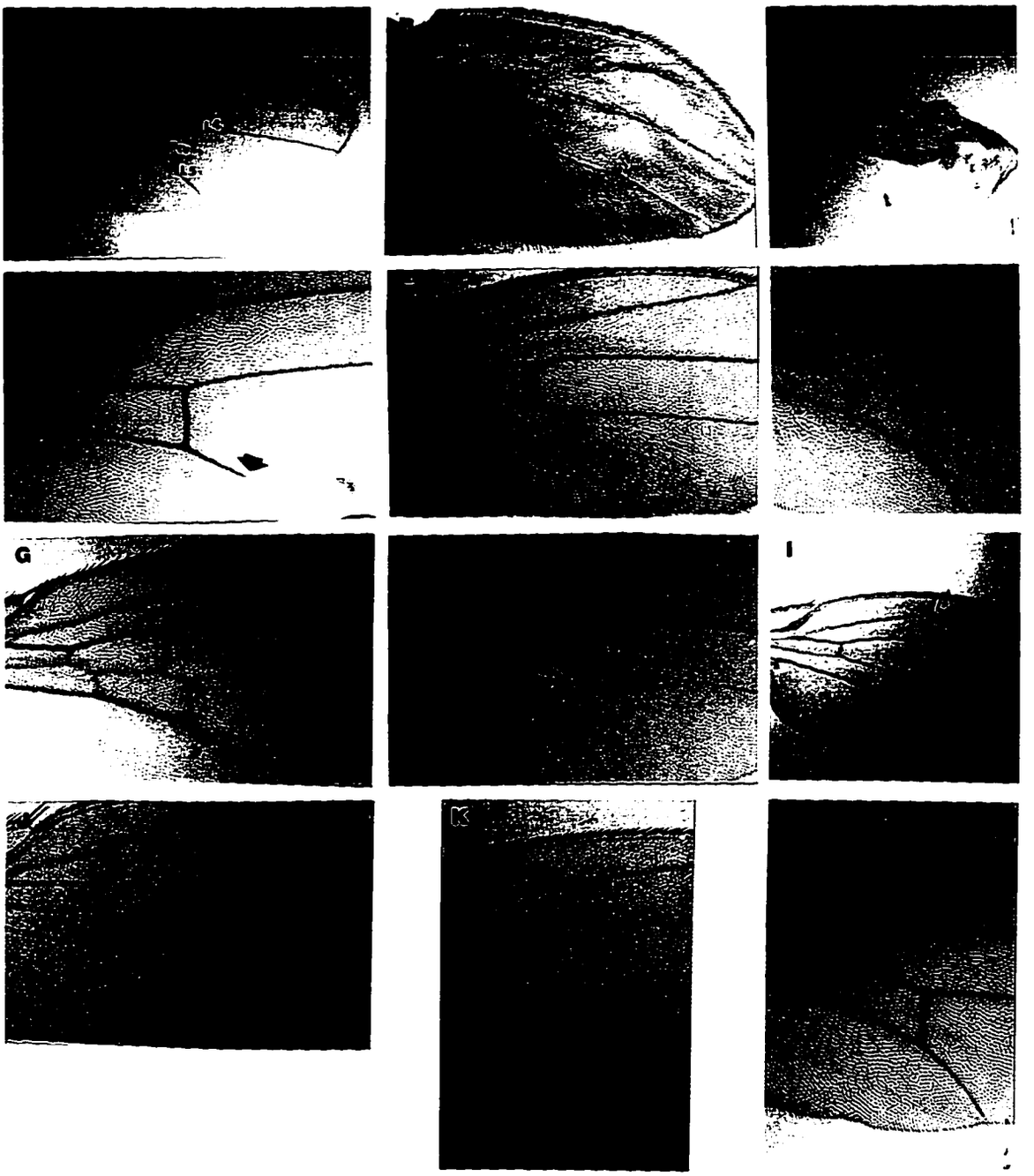


Table (5)

Wing phenotypes of *rugose* mutants

rugose alleles	% of defective wings at 17°C	% of defective wings at 24°C	% of defective wings at 29°C
rg^{P1}	3.38	2.58	11.32
rg^{P2}	0.00	4.25	21.11
rg^{P3}	38.29	19.05	22.50
rg^{P5}	14.80	3.50	12.19
rg^{P6}	0.00	2.12	24.18
rg^{P9}	4.34	2.40	2.63
rg^{X-1}	6.18	2.20	33.30
rg^{X-2}	6.19	5.87	11.45
rg^{X-3}	0.93	1.69	15.00
rg^{X-4}	1.85	1.58	8.73
rg^{X-5}	4.54	4.76	8.30
rg^{X-6}	2.29	0.00	3.83
rg^{X-7}	2.81	3.14	6.00
rg^{X-8}	0.00	0.00	1.60
rg^{X-9}	0.00	1.26	4.61
rg^{X-10}	2.81	3.20	24.18
rg^{X-11}	1.70	5.60	37.87
rg^{1}	0.00	18.50	23.67
CS	0.00	0.00	0.00
dhd	0.00	0.00	0.00

n= 100

Controls= Wild-type (CS) and *deadhead* (*dhd*)

Table (6)

***rugose* wing phenotypes (Flies were grown at 17°C)**

rugose alleles	% of incomplete L5	% of curved wings	% of shrunken wings	Total % of defective wings
rg ^{p1}		1.69	1.69	3.38
rg ^{P2}				0.00
rg ^{P3}	36.17		2.12	38.29
rg ^{p5}	6.17	4.93	3.70	14.80
rg ^{p6}				0.00
rg ^{P9}		4.34		4.34
r g $^{X-1}$			6.18	6.18
rg $^{X-2}$		3.87	2.32	6.19
rg $^{X-3}$			0.93	0.93
rg $^{X-4}$		1.85		1.85
rg $^{X-5}$		3.03	1.51	4.54
rg $^{X-6}$		2.29		2.29
rg $^{X-7}$			2.81	2.81
rg $^{X-8}$				0.00
rg $^{X-9}$				0.00
rg $^{X-10}$			2.81	2.81
rg $^{X-11}$		0.85	0.85	1.70
rg ^{1}				0.00

n= 100

Table (7)

***rugose* wing phenotypes (Flies were grown at 24°C)**

rugose alleles	% of incomplete L5	% of curved wings	% of shrunken wings	Total % of defective wings
rg ^{p1}	0.86	0.86	0.86	2.58
rg ^{P2}	1.25	3.00		4.25
rg ^{P3}	19.05			19.05
rg ^{p5}		3.50		3.50
rg ^{p6}		1.06	1.06	2.12
rg ^{P9}		2.40		2.40
rg $^{X-1}$	0.44	1.76		2.20
rg $^{X-2}$	0.58	5.29		5.87
rg $^{X-3}$		1.69		1.69
rg $^{X-4}$		1.58		1.58
rg $^{X-5}$		4.76		4.76
rg $^{X-6}$				0.00
rg $^{X-7}$		1.57	1.57	3.14
rg $^{X-8}$				0.00
rg $^{X-9}$			1.26	1.26
rg $^{X-10}$			3.20	3.20
rg $^{X-11}$	0.80		4.80	5.60
rg ^{1}			18.50	18.50

n= 100

Table (8)

***rugose* wing phenotypes (Flies were grown at 29°C)**

rugose alleles	% of defective cross veins	% of curved wings	% of shrunken wings	Total % of defective wings
rg^{P1}	7.54		1.88	11.32
rg^{P2}	16.90	1.40	2.81	21.11
rg^{P3}	17.50		5.00	22.50
rg^{p5}		2.44	9.75	12.19
rg^{p6}	24.18			24.18
rg^{P9}		2.63		2.63
rg^{X-1}	22.20	3.70	7.40	33.30
rg^{X-2}		2.29	9.16	11.45
rg^{X-3}	9.00	3.00	3.00	15.00
rg^{X-4}	4.85		3.88	8.73
rg^{X-5}			8.30	8.30
rg^{X-6}	1.27		2.56	3.83
rg^{X-7}	2.00		4.00	6.00
rg^{X-8}	1.60			1.60
rg^{X-9}	1.54		3.07	4.61
rg^{X-10}	8.06	3.22	12.90	24.18
rg^{X-11}	30.30	1.51	6.06	37.87
rg^{1}	18.42	1.31	3.94	23.67

n= 100

Table (9)

Phenotypes of *rugose* wing venation

rugose alleles	% of defective veins at 17°C (Incomplete L5)	% of defective veins at 24°C (Incomplete L5)	% of defective veins at 29°C (Extra cross veins)
rg^{p1}		0.86	7.54
rg^{P2}		1.25	16.90
rg^{P3}	36.17	19.05	17.50
rg^{p5}	6.17		
rg^{p6}			24.18
rg^{P9}			
rg^{X-1}		0.44	22.20
rg^{X-2}		0.58	
rg^{X-3}			9.00
rg^{X-4}			4.85
rg^{X-5}			
rg^{X-6}			1.27
rg^{X-7}			2.00
rg^{X-8}			1.60
rg^{X-9}			1.54
rg^{X-10}			8.06
rg^{X-11}		0.80	30.30
rg^{1}			18.42

n= 100

DISCUSSION

In this chapter the genetic and phenotypic characterization of *rugose*, a gene required for normal pattern formation in the *Drosophila* eye, has been described. Mutations in the *rugose* locus disrupt the normal pattern of the eye through effects in the cellular developmental events at the late third instar and early pupal stages. Cellular communication mechanisms employing ligands and receptors are required to specify cell identities. Mutations that affect these mechanisms either in the signaling or the reception process may lead cells towards incorrect developmental pathways. Inappropriate development and/or arrangement of the retinal cells cause roughening in the eye. Any defect that occurs between the third larval instar (at the onset of cell differentiation) to the mid-pupal life (when pattern formation is completed), results in a rough eye phenotype. In the third larval instar eye disc the five R cells of the precluster, R8, R2, R5, R3 and R4 are derived from the first mitotic wave ahead of the furrow, while all other remaining cells in the growing cluster are derived from the second mitotic wave behind the furrow (Tomlinson and Ready, 1988; and Cagan and Ready, 1989). Examination of *rugose* larval eye discs reveals normal cell division in the first and second waves of mitosis. This indicates that all retinal precursor cells are normally formed. Labeling with different antibodies (various R cell specific probes) has shown that in *rg* mutant eye discs the photoreceptor cell differentiation is normal. The results presented here show that neural cell determination in the developing retina

is not affected by the loss of *rg* function. The normal differentiation of R cells during the *rg* third larval instar suggested that *rg* gene may act at a later stage during the development of the eye. My results are consistent with previous studies by Renfranz and Benzer (1989) who also showed rg^1 in normal pattern formation at the third larval stage. At this stage only R cell differentiation takes place while cone, pigment and bristle cell differentiation is initiated at the end of the third instar stage.

***rugose* pupal eyes showed the development of fewer cone cells and extra pigment cells in the lattice. As a consequence, the bristles showed irregular arrangement in the defective lattice which gives the eye rough appearance. The defects seen in *rg* mutant eyes result from the failure of cone cells and pigment cell lattice to differentiate normally. On the other hand, there was no indication of any abnormal cell death that normally refines the pigment cell lattice. More likely, the decrease in cone cell number leads to an increase in the secondary and/or the tertiary pigment cells. Therefore, it is possible that loss/disruption of cell-cell communication in early pupation may result in cell transformation, where presumptive cone cells adopt the pigment cell fate/pathway. These cell fate choices may involve as yet undefined signal(s). Although mutations in *rg* caused defects in cone cells, mutants had the ability to develop the normal number of primary pigment cells that contact the cone cells. Primary pigment cells play an important role in the determination of the secondary and tertiary pigment cells that form the lattice (Cagan and Ready, 1989b). Perhaps, *rg* is required for cone cell development and subsequently signals from cone cells (through primary pigment cells) are required for proper pigment cell lattice formation.**

In spite of the normal development of the R cells in the third larval instar, the adult eyes showed abnormal number of R cells in some *rg* ommatidia. The arrangement of R cells is often irregular and some of these cells conceal from the ommatidia resulting in disturbed architecture of the adult eye. This may be explained by the defects found in the fenestrated basement membrane (FM) that supports the retina and the photoreceptor cells. Primary pigment cells which develop normally in *rg* mutant eyes do not extend to the floor of the retina. The secondary and tertiary pigment cell feet together with the cone cell feet contact and form the fenestrated basement membrane. Ommatidial bundles of eight R cell axons project through the FM at special holes which are ringed by adhesion molecules such as integrins (Longley and Ready, 1995). In their study, they found that retinal phenotypes change due to structure failure of the cone cell plate and adhesions of pigment cells. This plate is formed at the floor of the retina surrounding the axons exit to the optic lobe. In *rg* mutants non-uniform distribution of cone and pigment cell feet in the FM easily results in the formation of vacant regions in the filamentous membrane. Retinular cells which reside on top of these areas have no support at the floor of the retina. Thus, these cells could collapse and pass through the opened large gaps to reach the first optic ganglion, the lamina. In analogous situation, *strawberry notch* (*sno*) showed defective fenestrated membrane development, where some R cells drop to the brain (Coyle-Thompson and Banerjee, 1993). They reported that the loss of R cells is not due to improper determination of R cell fate but may be due to defects in cone or pigment cell recruitment.

Mutation in *rugose* have been shown to cause morphological and cellular defects not only in the eye but also in the wing formation. These results suggest that *rg* is also involved in the differentiation and/or survival of cells that form the wing and the wing veins. Moreover, the results clearly support the notion that *rg* protein is required to regulate cell fates in the developing eye and wing. Although *rg* function in the wing is not clearly understood, *rg* wing vein formation is very interesting. *rg* exhibits inhibition of wing venation at lower temperature, while it leads to the formation of extra veins at higher temperature. The loss-of-function phenotype at lower temperature is contrasted by the gain-of-function phenotype at higher temperature. This observation may suggest that *rg* may be involved in activating both positive as well as negative functions. For example, hypothetically *rg* may encode a protein phosphatase, which may activate or deactivate specific proteins in a signal cascade by dephosphorylation. Similarly *rg* could also encode a protein kinase. Reversible protein dephosphorylation by phosphatases is a well known mechanism that regulates cellular processes. Wassarman et al. (1996) suggested that protein phosphatase 2A regulates Ras1-mediated photoreceptor development positively and negatively by dephosphorylating factors at different steps of the Ras pathway. Interestingly, similar wing phenotypes are observed in mutations in genes that interact with *rugose*. These genes are known to function in a common pathway, ie. the EGF receptor mediated signal transduction pathway. Thus suggesting that *rg* may also function in the same or a related pathway (see chapter 4).

MATERIALS AND METHODS

γ-ray Mutagenesis and Complementation Tests

Three day old male w^{1118} flies were exposed to a γ-ray source (3000 rad) which cause random mutations. Several hundred of the irradiated males were crossed to homozygous virgin females from the original rg^1 stock that carry red eyes. F1 females were screened for random sex-linked recessive mutations. Forty eight rough eyed females were collected out of 16,100 flies. Each one of these females has a single mutagen-treated X-chromosome derived from its paternal parent and a *rugose* mutant X-chromosome derived from its maternal parent. The collected F1 virgin females were individually crossed to FM7c males (balancer). Males with white rough eyes were collected from the progeny, individually mated to virgin attached-X females [C(1)Dx,ywf] of M' cytotype and propagated as stable lines. Homozygous stocks were generated by crossing the mutant males to the wild-type virgin females and collecting F1 virgin females that carry the *rg* mutation on one X-chromosome and a wild-type X-chromosome. These virgins were mated to the isolated *rg* mutant males of the same allele and homozygous stocks were established. Two complementation tests were done. In the first test, the newly generated alleles were crossed to the wild-type virgins (Canton-Special) to determine if the obtained alleles have dominant mutations. In the second test, they were crossed to homozygous rg^1 virgins to confirm that the new mutations are allelic to *rugose*. The phenotype and the level of gene function of the new

alleles were determined by placing the mutant chromosome over a deficiency chromosome and comparing the rough eye phenotypes.

Isolation of P-element Induced *rugose* Mutations and Their Revertants

wdhd^{p8}/FM7p males which carry a single P-lac Z insertion in the deadhead gene, a maternal-effect gene required for early nuclear divisions (Salz et al., 1994), were crossed to C(1)Dx,ywf; Δ2-3sbTM3 females which provided the transposase. In this hybrid dysgenic cross, the male carries a transposable-P-element insertion in 4F1,2 region and the female's cytotype promotes P-element mobilization in the germ line of F1 flies. The second generation (F2) flies were screened for mutant males with rough eyes. Mutant flies that isolated from the hybrid dysgenic cross were individually mated to attached-X females of the P' cytotype to prevent further P-element mobilization. Homozygous stocks of two alleles (*rg*^{p2} and *rg*^{p5}) were generated. Complementation tests were carried-out to determine that the obtained alleles were due to mutations in the *rugose* locus. A reversion analysis was performed by hybrid dysgenesis. *rg*^{p2} and *rg*^{p5} males were crossed to C(1)Dx,ywf; Δ2-3 cytotype females. P-element were remobilized and separate revertant lines were isolated.

Scanning Electron Microscopic Studies

Adult flies were dehydrated in a series of ethanol for 48 hrs. Tissues were processed for critical point drying, mounted and coated with 25 nm thick gold coat.

Light Microscopic Studies

Adult flies were decapitated in 0.1 M Na Cacodylate buffer. Heads were fixed in 4% glutaraldehyde in buffer overnight at 4°C. Heads were washed in buffer and fixed in 2% O_SO_4 for 30min, then dehydrated, transferred into propylene oxide and infiltrated in Polybed 812 mixture overnight. The heads were embedded under a dissecting microscope and oriented for both tangential and transverse sections. Plastic blocks were sectioned by an ultra-microtome and 4μm sections were collected and stained in 0.5% Borax Toluidine blue stain.

Bromodeoxyuridine (BrdU)

Third larval instar eye discs were dissected in PBS and incubated in 5 mg/ml BrdU solution for 2hr. Eye discs were fixed in Carnoy's fixative for 30 min and then dehydrated in ethanol series. DNA was denatured in equal volumes of 4N HCl and 0.3% Triton-X in PBS. Tissues were washed in PBS and incubated in 10% goat serum in PBS-Triton-X for 30 min then incubated in 1:100 monoclonal anti-BrdU in PBS overnight. Tissues were washed in PBS-Triton-X and incubated in 1:200 Goat anti-mouse IgG HRP conjugated for 2 hrs at room temperature with shaking. Tissues were washed in the buffer then stained in diaminobenzedine (DAB) staining solution mounted in glycerol and examined under a light microscope.

Acridine Orange Stain

Eye discs were dissected in Ringer's solution and stained in $1.6x10^{-6}$ acridine orange in Ringer's. The tissues were washed in Ringer's solution, mounted in 30% glycerol in Ringer's and immediately examined under epifluorescence in a Zeiss microscope.

Immunohistochemistry

Neurospecific monoclonal antibody Mab22C10 staining procedure is followed after Zipursky et al. (1984). Third larval instar eye antennal discs were dissected and fixed in 2% paraformaldehyde in PBS for 15 min, then washed in 0.3% Triton-X in PBS and incubated for 3hrs in Mab22C10 diluted 1:1 with 0.3% Triton-X in PBS. Tissues were incubated in HRP conjugated secondary antibody and counterstained in DAB then mounted in glycerol.

Anti-Boss antibody staining (donated by Zipursky lab at UCLA) followed the procedure of Kramer et al. (1991) with some modifications. Eye discs were dissected and fixed in 4% paraformaldehyde in PBS for 30 minutes then washed and incubated in 1:500 anti-Boss antibody in PBS for 2hrs at room temperature. Tissues were washed and incubated in 1:200 Goat anti-mouse IgG HRP (obtained from Sigma) for 2hrs, then washed and stained in DAB staining solution and mounted in glycerol.

***Scabrous* (*Sca*) protein expression were tested by using a monoclonal antibody (obtained from Dr. N. Baker NYU). Eye discs were dissected and fixed in fresh PLP**

solution for 20 min then washed in 0.3% Triton-X in PBS and incubated in 50% *Scabrous* antibody in PBS overnight at 4°C. Tissues were washed incubated in (1:200) HRP conjugated secondary antibody for 3hrs. Tissues were washed and stained in DAB staining solution, then mounted in glycerol.

Enhancer Trap Lines

Homozygous enhancer trap line males, which have lac Z P-element insertion that encodes β-galactosidase, were crossed to homozygous *rg* mutant virgin females, two different lines were used in which lac Z P-elements were inserted into *sevenup* (*svp*) and *rhomboid* (*rho*) individually. The expression of β-galactosidase were localized by staining the eye discs with anti-β-galactosidase antibody. The eye discs were dissected and fixed in 4% paraformaldehyde in PBS for 20 min, then washed and incubated in (1:200) β-gal monoclonal antibody in PBS overnight at 4°C. The eye discs were washed and incubated in (1:200) Goat-anti-mouse IgG HRP for in PBS for 3hrs. Then stained in DAB staining solution and mounted in glycerol.

Staging The Pupa and Timing The Pupal Development

White prepupae were collected and considered Zero time of pupariation. They were maintained in room temperature (24°C) for 50hrs, then pupal eyes were dissected and studied. The time required for pupal development was detected by collecting white prepupae (Zero time) and maintaining them at room temperature till hatching. The

percentage of development at 50hr of pupal formation was calculated.

Cobalt Sulfide Stain

Cobalt sulfide staining protocol was modified from Melamed and Trujillo-Cenoz (1975) to stain cell membranes in 50hr pupal eyes. Pupal eye discs were dissected in 2% glutaraldehyde in water for 10 min, then washed briefly and transferred into 5% Cobalt Nitrate for 15 min. The tissues were rinsed in water for 5 sec. and transferred into a drop of 2% ammonium sulfide for 5 sec. then washed and mounted in glycerol.

Silver Stain

Silver staining procedure was modified from Meyerowitz and Kankel (1978). Adult heads were decapitated and fixed in Bowin's solution, washed in water and dehydrated in ethanol series. Heads were transferred into xylene followed by a mixture of xylene/paraffin then into 100% paraffin at 60°C for 1hr. Heads were embedded and oriented in fresh paraffin. Sections were cut 5μm each and dewaxed in xylene then dehydrated. Sections were stained in Blest's silver mixture for 16hrs at 37°C then developed into hydroquinon sodium sulfite developer for 9 min at 55°C. Sections were washed then toned in 0.75% $AgNO_3$ for 20 min under bright light. They were transferred into 2% oxalic acid followed by 5% sodium thiosulfate 5min each. Tissues were dehydrated and then dried in xylene and mounted in premount media.

CHAPTER III

DEVELOPMENTAL STUDIES ON THE *rugose* COMPOUND EYE

INTRODUCTION

In the *Drosophila* compound eye, cells differentiate by sequential induction involving cell-cell interactions. Genetic mosaic studies have clearly established that the photoreceptors (R cells) and pigment cells are not determined by cell lineage (Ready et al., 1976; Lawrence and Green, 1979). Clonal analysis by Wolff and Ready (1991a) suggested that cone cell determination is also probably specified by cell-cell interactions. The nature of these interactions is not very well understood. Cone cells in addition to secreting the lens of the compound eye also play a role in the formation of the fenestrated basement membrane that shapes and supports the retina. In the pupal stage cone cells together with the primary pigment cells produce an extracellular secretion called the pseudocone. Cone cells (also called Semper's cells) extend from the pseudocone posteriorly to the floor of the retina. They terminate as flat feet that contain ommachrome pigment granules (Cagan and Ready, 1989b). The cone cells and the photoreceptor cells are surrounded by pigment cells. Two primary pigment cells enclose the cone and R cells from the anterior and posterior and isolate them from the external lattice. The primary pigment cells contain large brown ommachrome granules resemble those found in the cone cell feet. Secondary and tertiary pigment cells form

the hexagonal lattice that shapes the ommatidia. The secondaries and the tertiaries contain small red pteridine pigment granules which are distributed variably along the length of the cells. The feet of these cells flatten and form the fenestrated basement membrane (Cagan and Ready, 1989b; Wolff and Ready, 1993). Cone cell development begins immediately after the differentiation of the R7 cell, the last photoreceptor cell to differentiate. So far, very little is known regarding the molecular basis of cone and pigment cell specification in the compound eye. Some of the genes that are required for proper specification of the photoreceptor cells have also been shown to affect the specification of cone cells. The cone cell precursors are recruited from five pluripotent cells that express the Sevenless protein (Sev). Only one cell that contacts the Boss-expressing R8 cell develops into an R7 cell. The other four cells adopt a nonneural fate and become lens-secreting cone cells. Ectopic expression of either Sev or Boss in these cone cell precursors results in their transformation into extra R7 cells (Zipursky and Rubin, 1994). On the other hand, the outer photoreceptor cells R3, R4, R1 and R6 express Seven-up (Svp), a steroid hormone receptor (Mlodzik et al., 1990b). Low doses of ectopic Svp in cone cells results in their transformation into R7 cells, while high doses results in the conversions of both the R7 and cone cells into outer photoreceptor cells (Hiromi et al., 1993; Begeman et al., 1995; Kramer et al., 1995).

In the previous chapter my results showed that in the eye, *rg* gene function is specific for cone cell differentiation and that *rg* mutations do not affect the photoreceptor cell determination. To precisely determine the developmental stage at which the developmental defects caused by the *rg* mutations appear, I have carried out

a developmental analysis of the *rg* mutant phenotype employing light and electron microscopic methods. In these developmental studies I have followed retinal formation at various developmental stages with expectation that these studies may help to define a critical time period during which *rg* gene product is required for cone cell development.

RESULTS

Eye Development During The Third Larval Instar Stage

Eye development begins during the third larval instar when the morphogenetic furrow (MF) sweeps the eye-imaginal disc from the posterior to the anterior. Immediately posterior to the MF, bundles of differentiating photoreceptor cells (R cells) were observed. Electron microscopic studies showed normal differentiation of R cell clusters in both wild-type and *rugose* mutant eye discs. The nuclei of the photoreceptor cells are arranged in two layers, an apical layer which contains the R8 and R2-R5 nuclei, and a basal layer that includes R1, R6, R7, cone cells, pigment cells and bristle cells nuclei. Each R cell occupies a precise position in the developing ommatidium. The nucleus of R8 cell was found between R2 and R5 nuclei. R3 and R4 nuclei occupy the polar position and contact R2 and R5 respectively. The five R cells in the precluster form a circle and cell membranes of adjacent cells contact [Fig. 13A,B]. The R8 nucleus is the first to move basally followed by R2 and R5 nuclei. R6, R1 and R7 nuclei start to rise from the basal region to the apical surface in order. The R8 cell occupies

the central position and the other R cells reside in specific positions around its border [Fig. 13C,D]. Once the nucleus of R7 rise to the apical surface in between R1 and R2, the mature cluster of eight R cells is established [Fig. 13E,F]. Further development in the eye continues during the pupal stages.

Early Pupal Development

Early in pupal formation the disc reverses its invagination and retains its connection with the optic lobe that moves towards the center of the eye. Development of the eyes was studied at zero time of pupal formation, (as soon as the third larvae became white prepupae) by using both light and electron microscopic preparations. In the white pupal eye, two stages of cone cell development were observed. Once the R7 nucleus completes its migration to the apical surface of the retina, two cone cell nuclei rise up from the basal region and flank the R cell cluster from the anterior and posterior developing the two cone cell stage. The anterior cone cell contacts R1, R2 and R3 while the posterior cone cell contacts R4-R7 [Fig. 14A]. The nuclei of R3, R4 and R7 are closer to the surface and R1, R2, R5, R6 and R8 located basally. Soon after two other cone cell nuclei rise from the basal to the apical region forming the four-cone-cell stage [Fig. 14B]. At this stage the nuclei of R3, R4 and R7 withdraw back into the basal region, the R4 cell is no longer in contact with the R8 cell, and the newly developed cone cells occupy the polar and the equatorial positions. The polar

Figure 13: Electron micrographs showing the apical cross sections in the wild-type (A, C and E) and *rg*$^{x-6}$ (B, D and F) third larval instar eye discs. (A) and (B) showing the differentiation of five R cell nuclei. (C) and (D) showing seven R cell nuclei. (E) and (F) showing complete clusters of eight R cell nuclei. No differences were found between the wild-type and *rg* mutant third larval instar eye discs. Anterior to the right. 10,000X

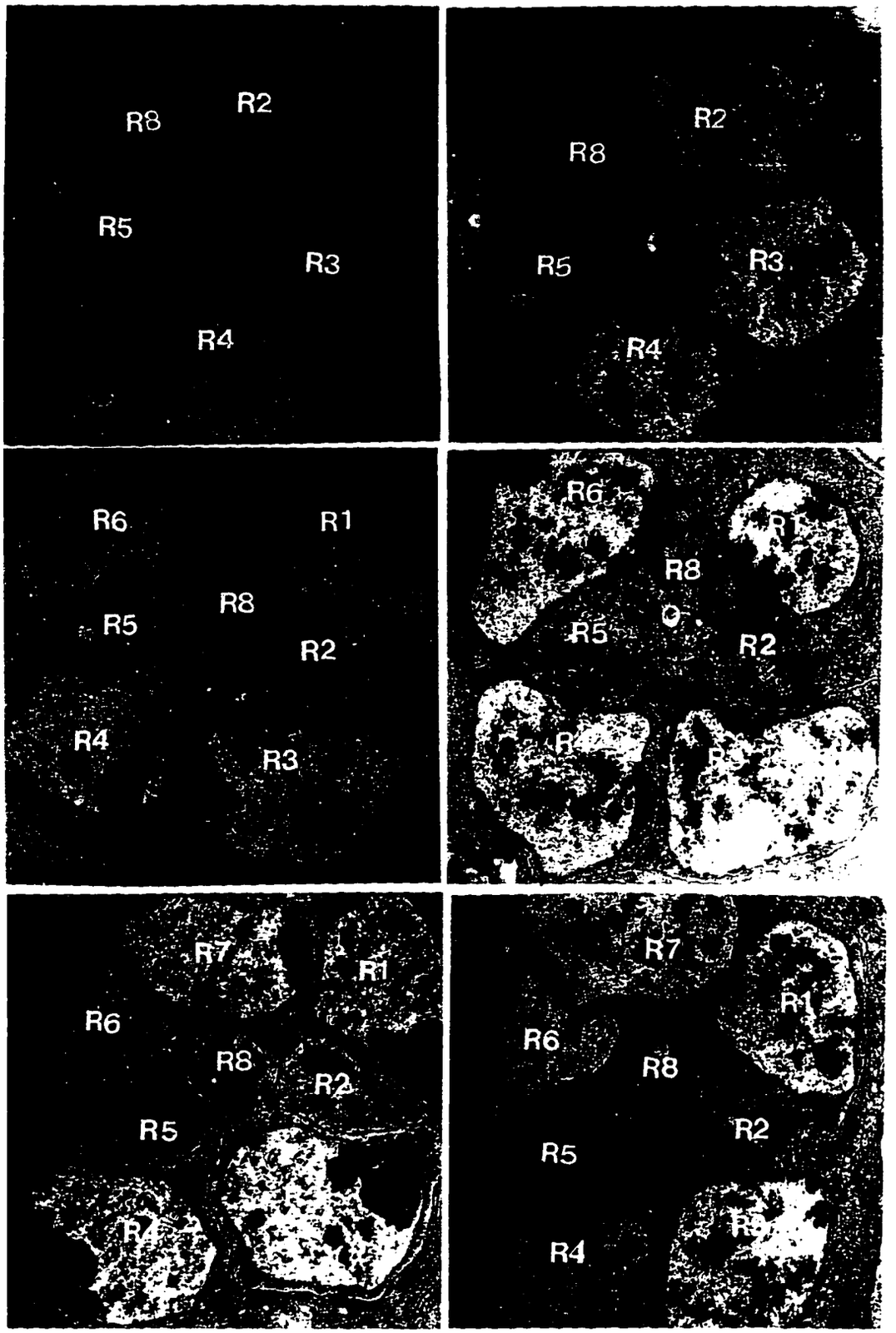
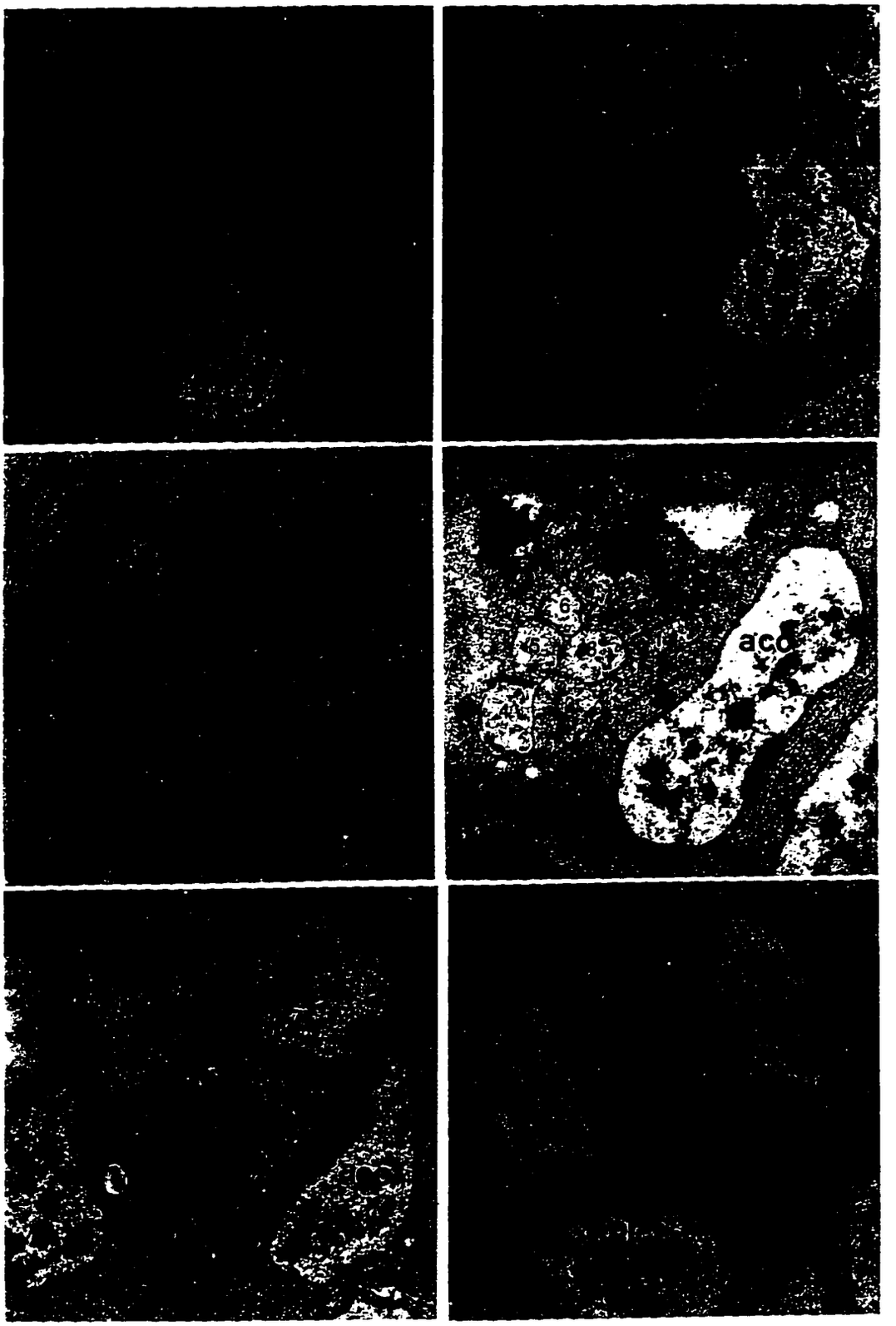
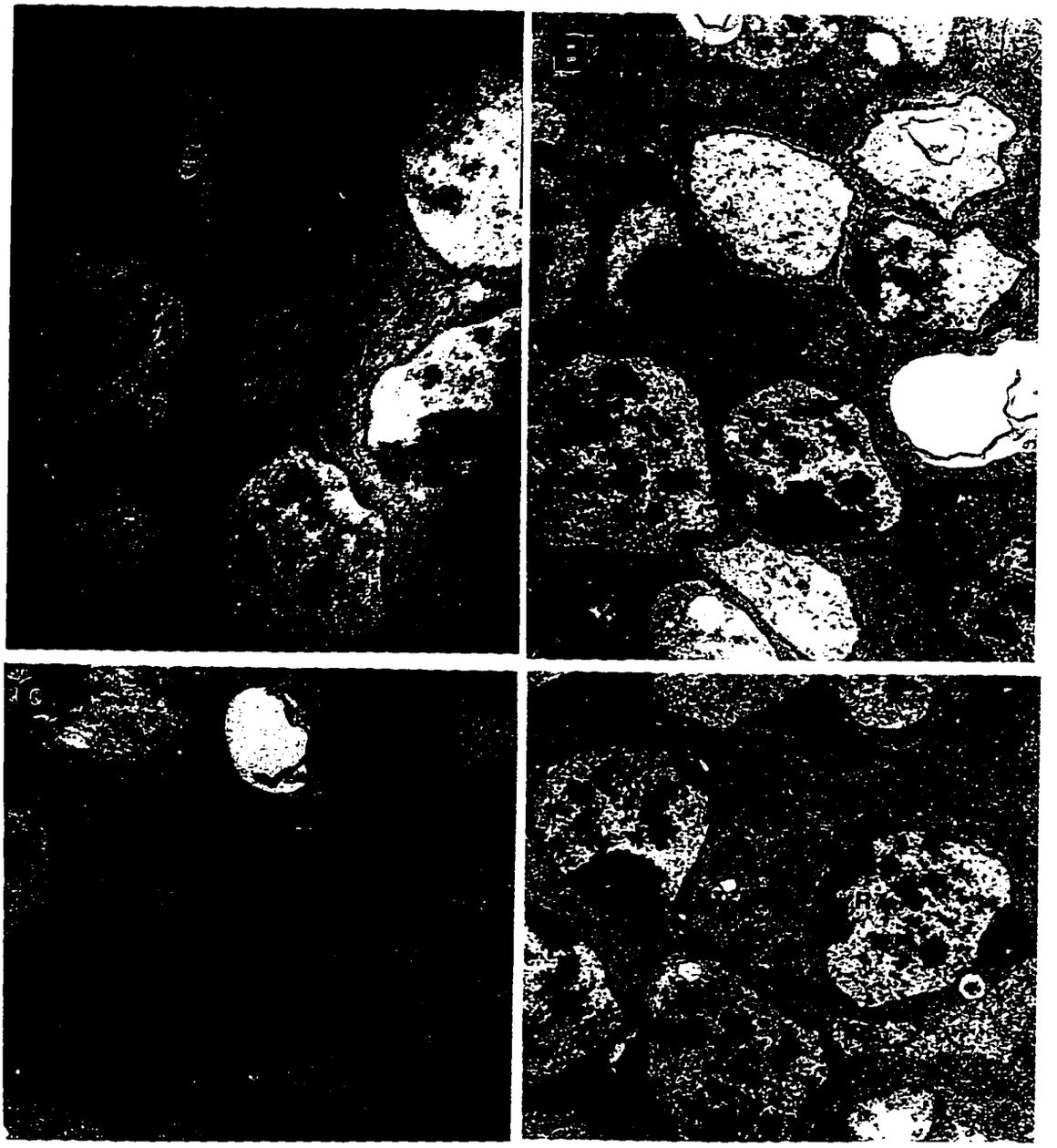


Figure 14: Electron micrographs showing apical cross sections in the wild-type and *rugose* mutant white pupal eyes (at 0 time of pupal formation). (A) Wild-type two cone cell stage showing eight R cell nuclei, an anterior cone cell (acc) and a posterior cone cell (pcc). (B) In the wild-type four cone cell stage, the eight R cell nuclei migrate basally. A polar cone cell (plcc) and an equatorial cone cell (ecc) differentiate. (C) rg^{x-3} showing no cone cells. (D) rg^{x-6} showing one cone cell (acc). (E) rg^{x-6} showing two cone cells, the anterior and posterior cone cells. (F) rg^{x-6} showing three cone cells, no equatorial cone cell. Anterior to the right. Asterisks show the positions of the missing cone cells. 10,000X



cone cell contacts R3 and R4 while the equatorial cone cell contacts R7. The four cone cells spread over the apical projections of the photoreceptor and reside on the ommatidial surface. The four-cone-cell stage is the most advanced cellular development accomplished in the white pupal eyes. In *rugose* mutant eyes I have observed variable number of cone cells per ommatidium. Depending on the *rugose* (*rg*) gene dosage, *rg* alleles show different defects. However, all *rg* alleles showed a decrease in cone cell number. I have observed variation in cone cell number between zero and four cone cells [Fig. 14C, D, E and F]. No more than four cone cells were ever seen in *rg* mutant eyes. All eight photoreceptor cells within the cluster project their axons to the basal region. The axonal bundle passes to the deeper tissue where only non-neural cell nuclei are located. In the wild type, eight axons forming an axonal bundle can be seen surrounded by pigment cell nuclei. 20hrs after pupal formation, the axonal bundles are surrounded by cone cell feet. In *rg* mutants some axonal bundles were surrounded by less than four cone cell feet [Fig. 15A,B]. The nucleus of a photoreceptor cell is located within the axonal bundle at the bottom of the retina [Fig. 15C,D]. Moreover, the arrangement of pigment cell nuclei was abnormal in *rg*. In some areas two axonal bundles were seen close to each other and no pigment cell nuclei were found in between the two bundles. In other areas, comparing the number of nuclei that surround the axonal bundles, more nuclei were found in the *rg* mutant eyes. This finding may support the probability of extra pigment cell development in the lattice. These results are based on electron microscopic studies.

Figure 15: Electron micrographs showing basal cross sections of 20hr pupal eyes. (A) Wild-type showing four cone cell feet lie to the sides of the axonal (ax) bundle and are surrounded by non-neuronal cell nuclei. (B) *rugose* mutant (rg^{x-10}) showing three cone cell feet. (C) and (D) rg^{x-10} showing the nuclei of R cells come to reside basally in the axonal bundle level. A and B (10,000X), C and D (13,000X).



Pupal Eye Development Between 50 and 60 hr

At the early pupal eye development, cells that contact the anterior and the posterior cone cells respectively becomes anterior and posterior pigment cells. Two primary pigment cells which contain large ommachrome pigment granules differentiate and enwrap the four cone cells. Cells which contact primary pigment cells develop as secondary and tertiary pigment cells that contain smaller pteridine pigment granules. The cell contacts two primary pigment cells of two adjacent ommatidia becomes a secondary pigment cell while the cell contacts three primary pigment cells becomes a tertiary pigment cell. These cells form the lattice that evenly spaces the ommatidia. In the differentiating lattice two to three extra cells per ommatidium are eliminated by programmed cell death. Cell death begins by the time of the primary pigment cell differentiation (Ready, 1989; Cagan and Ready, 1989b; Wolff and Ready, 1991). Cell-cell contact is an important aspect that governs cell death. In the lattice cell contact with the primary pigment cells is essential for survival. Cells with equal contact with the primary pigment cells compete for the position and the loser dies (Wolff and Ready, 1991). For example, two cells in the presumptive horizontal secondary pigment cell position compete, one cell is able to determine its fate while the other is eliminated by cell death. Similarly, three cells in the tertiary pigment cell position compete and only one cell establishes its fate and the other die. By sixty hours of pupal formation the cellular development of the retina is completed. The mechanosensory bristle group development is distinct from the

ommatidial cells development. The four cells that form the bristle group comes from a single ancestor cell. The tormogen cell secretes the socket and the trichogen cell produces the bristle. These two cells exist only in the pupal life. The sensory neuron and the thecogen cell with a supportive glial cell remain in the adult eyes. The bristle division begins at the center of the eye and spreads radially. Bristles adopt their final positions in the eye after elimination of the undifferentiated cells through programmed cell death (Cagan and Ready,1989b).

Fifty hours after pupal formation both of the wild-type and the *rugose* mutant eyes were dissected and stained with cobalt sulfide stain. This stain highlights the cell membranes of the cone and pigment cells and elucidates the pattern formation at the apical surface. The typical arrangement of completely developed retina was observed in the wild type 50hr pupal eyes [Fig. 16A]. In *rugose* mutant eyes variable number of cone and pigment cells were observed. The most severe defects were observed in rg^{x-3} which is a null allele, a complete loss of function mutation. In rg^{x-3} fifty hour pupal eyes few cone cells were found. A great number of ommatidia completely lack cone cells and others contain one or two cone cells only. The outer lattice shows multiplication of pigment cells with abnormal arrangement which makes cell counting a difficult task. These defects highly disturb the pattern formation of the eye [Fig. 16B]. Another *rugose* allele, rg^{x-6} showed mostly two cone cells per ommatidium and occasionally one or three cone cells [Fig. 16C]. The orientation of these cells looked abnormal as one cannot easily determine the position of each particular cell. In addition, the bristles arrangement is disrupted due to the irregular development of the

lattice. Although the primary pigment cell development appeared to be normal in *rugose* mutants, the outer lattice surrounding the ommatidia was greatly affected by the mutation. It appears that extra cells are located in the position of the secondary and tertiary pigment cells as in rg^{p5} [Fig. 16D].

The Cut protein has been shown to be specifically expressed in cone cell precursors and as such serves as a very useful marker to identify cone cells in the developing eye (Blochlinger et al., 1990). In order to localize cone cells in the developing eye, sixty hours pupal eyes were labeled with cut antibody. In the wild-type four cells showed the staining, while *rugose* mutant eyes showed different number of cells that express protein against cut antibody [Fig. 17A,C,E,G]. Thus, some cone cells failed to differentiate in *rugose*, consistent with the electron microscopic observation. Similarly, MAb3F11 (Venkatesh et al.,1985), antibody which labels cell membranes of both cone and pigment cells was also used as a marker to localize differentiating cone and pigment cells. This labeling showed the irregular arrangement of the cone and pigment cell lattice in 60hr *rg* mutant eyes [Fig. 17B,D,F,H,I,G]. Electron microscopic studies of sixty hour pupal eyes showed abnormal number of pigment cells in the lattice. Some R cells from two neighboring ommatidia were touching due to the lack of pigment cells in between the ommatidia [Fig. 18A,B]. While other ommatidia were separated by extra pigment cells. At the bottom of the retina axonal bundles were surrounded by one or two extra pigment cells than those in the wild-type [Fig. 18C,D].

Figure 16: Light micrographs of the apical surface of 50hr pupal eyes stained with Cobalt Sulfide stain. (A) Wild-type showing normal development of four cone cells/ ommatidium in the retina. (B) *rg*$^{x-3}$ showing the development of one or no cone cell/ ommatidium and severe defects in the lattice. (C) *rg*$^{x-6}$ showing mostly two cone cells and abnormal bristle positions. (D) *rg*^{p5} showing one or two cone cells/ ommatidium and extra secondary and tertiary pigment cells in the lattice. 1000X

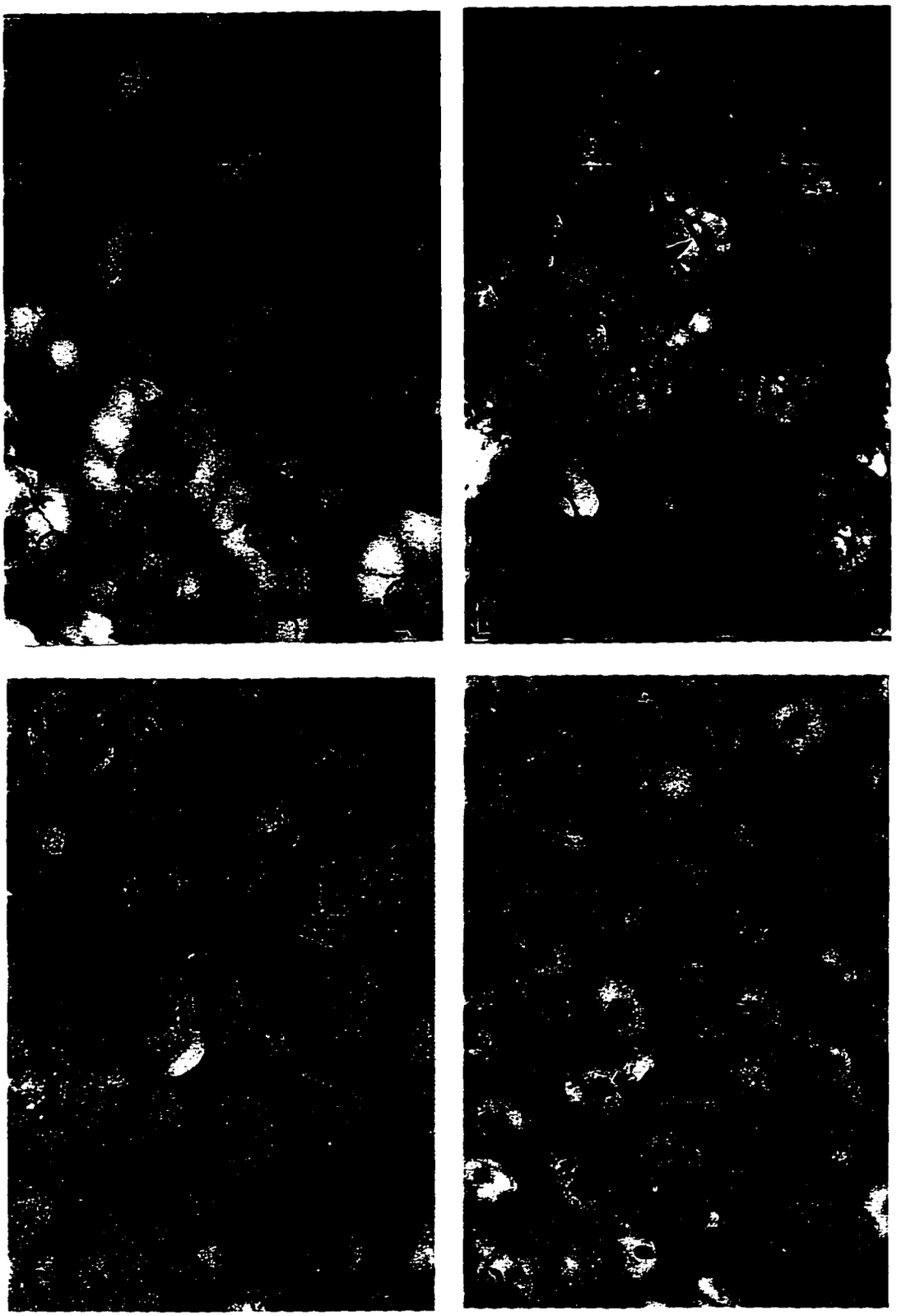


Figure 17: Fluorescein micrographs showing the apical surface of 60hr pupal eyes. A, C, E and G labeled with cut antibody, while B, D, F, H, I and J labeled with MAb 3F11. (A) and (B) Wild-type pupal eyes showing four cone cells. (C) and (D) rg^{x-10} showing three and four cone cells. (E) and (F) rg^{x-6} showing two cone cells. (G) and (H) rg^{x-3} showing one, two or no cone cells. (I) Wild-type showing normal hexagonal lattice. (J) rg^{x-6} showing abnormal lattice. 1000X

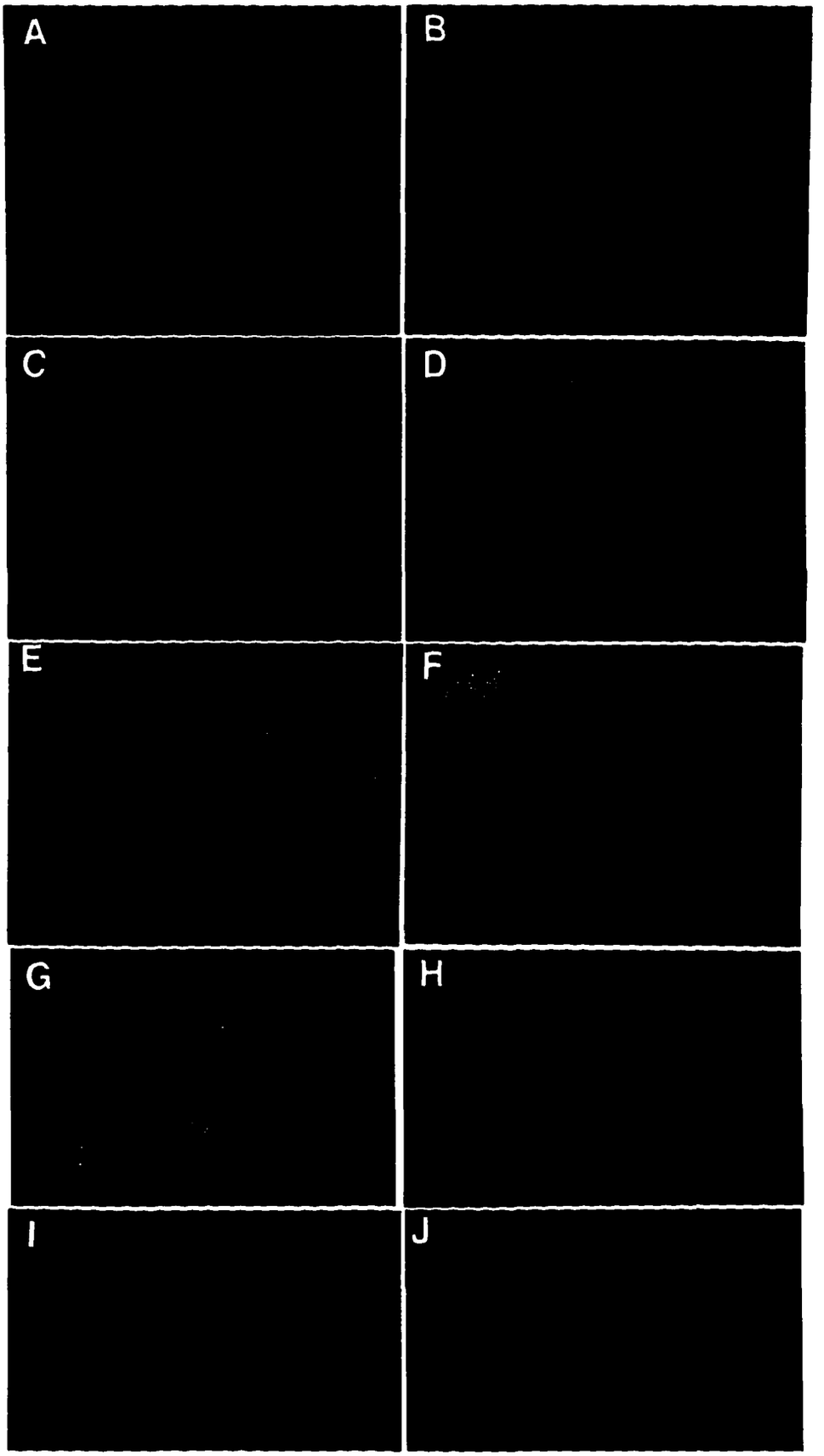
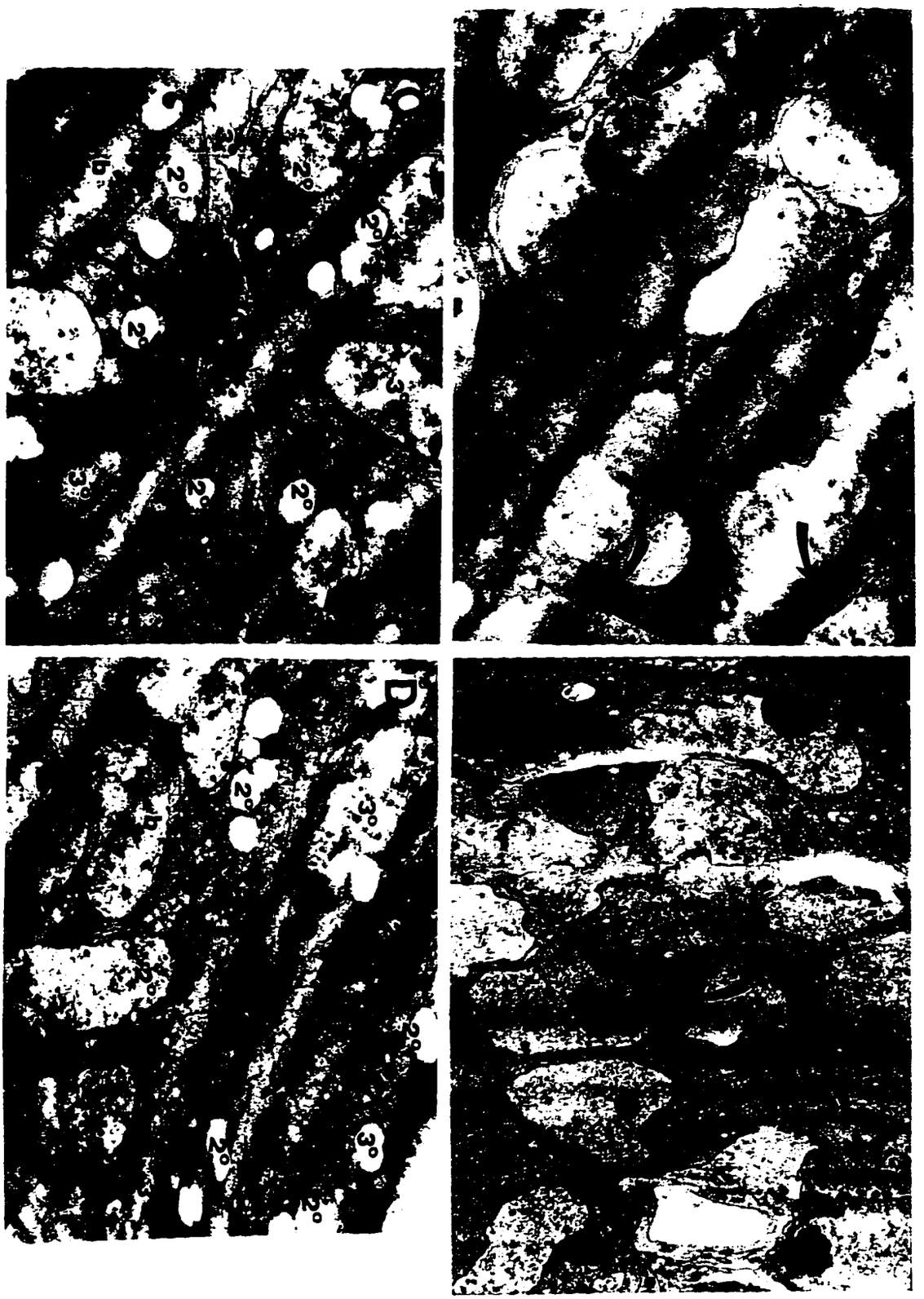


Figure 18: Electron micrographs of 60hr pupal retinas. (A) A wild-type cross section showing 7R cell ommatidium surrounded by pigment cell lattice that separate adjacent ommatidia. (B) *rg*^{p5} showing the lack of pigment cells between two adjacent ommatidia. (C) and (D) the bottom of 60hr pupal retinas. (C) In the wild-type axons of R cells in the center (small asterisk) are surrounded by six secondary pigment cells (2^{O}) and three tertiary pigment cells (3^{O}). (D) *rg*^{p5} showing the central axons are surrounded by 2^{O} and 3^{O} pigment cells including one extra cell (large asterisk). Bristle group (b). 10,000X



The Structure of The Adult Eye

During the late pupal stage, sixty to one hundred sixty hours after pupal formation, specialized cellular structures and products such as rhabdomeres, lenses and pigment granules are established. The depth of the retina increases about five times and the ommatidial cells elongate forming the typical shape of the adult eye. The primary pigment cells remain at the apical surface and do not reach the floor of the retina (Cagan and Ready, 1989b). The secondary and tertiary pigment cell feet extend under the photoreceptor and the cone cell feet forming flat plates that develop the fenestrated basement membrane. This membrane together with subretinal cells supports and shapes the inner surface of the retina. The subretinal cells originate from undifferentiated cells in the third larval eye imaginal disc. During the third larval stage undifferentiated cells that lose contact with the apical surface fall beneath the epithelial membrane forming a population of subretinal cells. The fenestrated membrane is formed above the subretinal cells. The lens and pseudocone structures are secreted by the cone and pigment cells. The cone and primary pigment cells secrete light strands towards their apical surface. Soon after, the secondary pigment cells secrete dark strands. Both light and dark strands form the lens above the ommatidium. At about 110 hours, cone cells secrete gelatinous product that form the pseudocone in between the lens and cone cells. This developmental manner creates the typical pattern of the eye surface. The lens becomes thick on top of the cone and primary pigment cells and thins at the junction between one lens and the other (Cagan

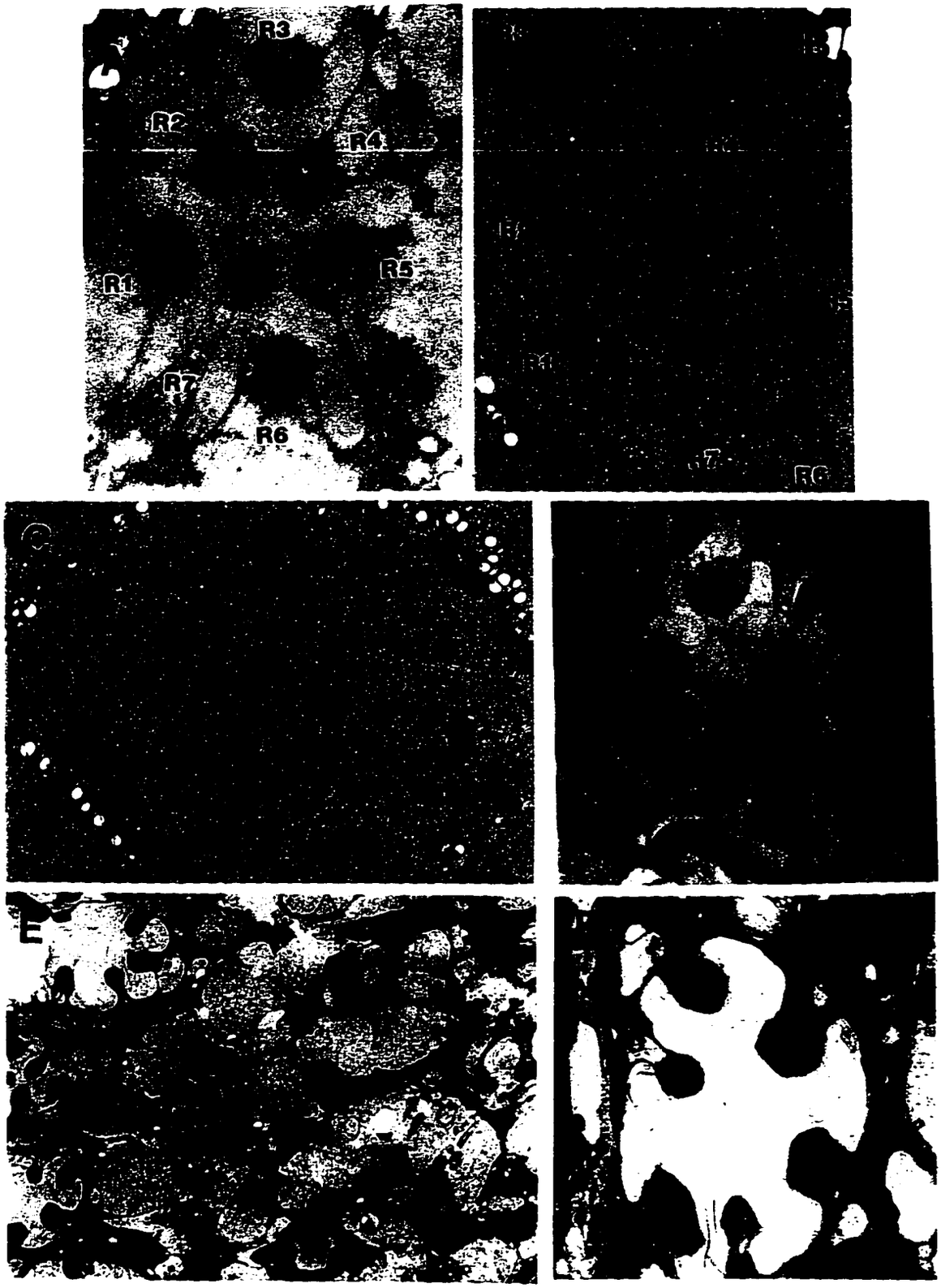
and Ready, 1989b).

Electron microscopic study of the adult eyes showed abnormal development of the rhabdomeres. In the *rugose* mutant eyes fusion or split of the rhabdomere structure were observed [Fig. 19D]. Abnormal arrangement of the photoreceptor cells in the ommatidium was found. This causes some R cells to occupy a larger niche pushing others in a smaller space [Fig. 19A,B]. Moreover, *rg* eyes showed fusion of two ommatidia where the R cells of adjacent clusters contact due to the loss of the pigment cell lattice in between [Fig. 19C]. These defects in the rhabdomere structure, the R cells positions, and the irregular arrangement of the pigment cell lattice result in the rough appearance of the *rg* adult eye [Fig. 19E,F].

DISCUSSION

Cells in the *Drosophila* eye are directed into their developmental pathway by signals that facilitate communication between cells and by positional cues. A mutation can cause defects in the cellular pathway and guide them into inappropriate fates. In *sevenless* mutation the cell in R7 niche is misdirected into the cone cell pathway (Tomlinson and Ready, 1986 and 1987b). Either the cell is not capable of receiving the correct developmental cues or it is unable to respond properly. Consequently, the cell follows inappropriate developmental pathway and adopts a different cell fate. These

Figure 19: Electron micrographs of the apical tangential sections through ommatidia of adult flies. (A) In the wild-type the six outer photoreceptors (R1-R6) are arranged in an asymmetrical trapezoid pattern and the central R7 cell has a smaller rhabdomere. (B) *rg*$^{x-6}$ showing that the R4 cell occupies a larger area than that in the wild-type, leading to abnormal arrangement of the R cells in the ommatidium. (C) *rg*$^{x-6}$ showing fusion of two ommatidia and abnormal arrangement of the rhabdomeres. One of the ommatidium shows only 5R cells. (D) *rg*$^{x-3}$ shows split of the R7 rhabdomere. (E) A tangential section in *rg*$^{x-3}$ eye shows disrupted ommatidia and lattice. (F) *rg*$^{x-3}$ ommatidium showing abnormal number and size of the rhabdomeres. A, B, D and F (10,000X); C (6,800X); E (5,200X).



events may lead to a duplication of certain kind of cells and/or the lack of others.

Although the identity of a cone cell can be determined by its position, the lack of one or more cone cell may entirely develop novel positions. The electron microscopic studies have shown the exact positions of the differentiated cone cells in the white pupal eyes. In the *rg* mutant eyes, when a single cone cell is missing, it is particularly the equatorial or the polar cone cell. Thus the ommatidia that include three or two-cone-cells have completed the two-cone-cell stage but not the four-cone-cell stage. Other ommatidia, that showed one or no cone cell, neither completed the first nor the second stage of cone cell development. Depending on the *rg* gene dosage there is a temporal interruption of cone cell development. This may suggest that the temporal expression of the *rg* protein is required at the time of the white pupal development. Reducing the Rugose activity at a specific interval causes reduction in cone cell number. It is possible that Rugose is required for the cell to receive an inductive signal for selecting the proper fate or it is essential for the cell to respond to that signal. Studying the nature of the *rugose* gene and its protein product will allow more understanding to the role of *rg* in cell fate determination.

In the lattice, the cell that contacts two primary pigment cells from two adjacent ommatidia develops as a secondary pigment cell. Whereas, the cell that contacts three primary pigment cells of three close by ommatidia becomes a tertiary pigment cell. The establishment of secondary and tertiary pigment cells is based on the pattern of contact they make with the neighboring primaries (Wolff and Ready, 1993). The results have shown normal primary pigment cell development and defective lattice.

Perhaps the primary pigment cells lost their ability to limit the number of the secondary and tertiary pigment cells. These defects in the lattice formation are directly or indirectly due to the reduced Rugose activity. The cobalt staining as well as the MAb3F11 have labeled the pigment cell membranes and showed accumulation of pigment cells in the retina. The observation of extra pigment cells in the lattice could be further investigated by detecting whether pigment cell-specific markers are expressed in those extra cells. So far, there is no direct evidence regarding the relationship between the missing cone cells and the extra pigment cells in the ommatidial lattice. However, we cannot rule out the possibility that due to the lack of functional Rg protein in the *rg* mutants, the cone cell precursors may be directed towards a pigment cell fate. This is also supported by the results that showed normal cell death and the regular size of the eye. The structure of the adults eyes of *rg* mutants showed abnormal backaging of the ommatidia and abnormal arrangement of photoreceptor cells. These defects results from the abnormal number of cone and pigment cells which affects the pattern formation of the eye.

MATERIALS AND METHODS

Staging of Pupae

Both of the wild-type (Canton-Special) and *rugose* mutant flies were raised at

room temperature on standard corn meal-agar food. As soon as the late third larvae became white prepupae, they were collected and considered 0-hr pupae. White prepupae were maintained at room temperature for 20hrs, 50hrs or 60hrs, then the pupal eyes were dissected.

Electron Microscopy

Tissues were prepared for Transmission electron microscopic studies essentially as described in Longley and Ready (1995) with few modifications. Both wild-type and mutant eyes were dissected in the fixative which contains 1% formaldehyde and 0.88% glutaraldehyde in 0.1M sodium cacodylate buffer (PH 7.4). Eyes were placed into few drops of fresh fixative for 4hrs at room temperature. They were then incubated overnight in 1ml of 1% tannic acid in fixative. Samples were washed in the buffer for 30min., incubated for 2hrs in 2% osmic acid in the buffer and washed three times in distilled water 10min each. Tissues were incubated overnight in 2% uranyl acetate. Dehydration took place in a series of ethanol, 50, 70, 80, 90, 100% 5min each. Tissues were then transferred into 1:1 ethanol and propylene oxide mixture for 10min followed by 15min in 100% propylene oxide. The eyes were then incubated into 1:1 propylene oxide and EMbed 812 mixture for 4hrs, then into 100% EMbed 812 overnight. The eyes were embedded and oriented in 100% EMbed 812 and DMP-30 accelerator and kept for polymerization in 60°C oven for three days. Samples were sectioned by using Ultra microtome and collected onto 300 mesh copper-coated grids.

Sections were viewed in a Phillips-300 electron microscope.

Light Microscopy

Cobalt sulfide staining modified from Melamed and Trujillo-Cenoz (1975), were used to visualize the apical surface of 50hr pupal eyes. Eyes were dissected in 2% glutaraldehyde in PBS buffer for 10min, then washed briefly in distilled water and transferred into 5% cobalt nitrate for 15 min. The tissues were rinsed in water for 5 sec and then transferred to a drop of 2% ammonium sulfide for 5sec. The tissues were washed in distilled water for 5min and mounted in glycerol.

Staining 60hr pupal eyes with Cut antibody (Blochlinger et al., 1990). Eye discs were dissected in PBS buffer and fixed in 4% paraformaldehyde then incubated in (1:100) Cut antibody for 3hrs. The tissues were washed in PBS and incubated in FITC-conjugated anti-mouse for 2hrs then washed and mounted in pheneline diamine glycerol.

MAb3F11 was diluted 50% in PBS-TX and used to label 60hr pupal eyes for 3hrs. Tissues were then incubated in goat anti-mouse FITC conjugate for 1hr then washed and mounted in pheneline diamine glycerol.

CHAPTER IV

GENETIC INTERACTIONS OF *rugose*

INTRODUCTION

Studies of genetic interactions have played an important role as to the understanding of cellular pattern formation during the development of *Drosophila*. Pattern formation is directed by gene products that are involved in cell-cell interactions. Frequently, cell specification is determined by the interaction of several genes participating in a particular signaling pathway. Genetic interactions are important as they regulate gene functions in a signaling cascade. Recently, molecular genetic studies on the determination of the photoreceptor cells (R cells) in the compound eye have demonstrated the importance of signal transduction mechanisms in the development of the *Drosophila* retina. In the *Drosophila* eye, the activation of receptor tyrosine kinases (RTKS) is a critical step in the signaling pathway. This step initiates a signal cascade that leads to cell fate determination. One of the most important pathways is the epidermal growth factor and its receptors which regulate cellular signal transduction cascade involved in cell growth, development, and differentiation (Brunner et al., 1994). The *Drosophila* homologue of the mammalian EGF-r (DER) is a receptor tyrosine kinase (RTK) which plays an essential role in cell-cell interactions during development. DER is required in the development of

photoreceptor cells, the formation of wing veins and the establishment of the embryo polarity (Xu and Rubin, 1993 and Brunner et al.,1994). The activation of DER results in the initiation of tissue and cell-specific signal transduction cascade involving Ras and MAP kinases. Information between cells are transduced when membrane bound receptors are activated by extracellular ligands. This ligand-receptor activation involves the interaction of particular gene products in the pathway. Several studies have identified genes that interact and regulate signal transduction cascade leading to specification of photoreceptor cells. The interaction of *sevenless* (*sev*) a receptor in the R7 cell and *bride of sevenless* (*boss*) a ligand made by the R8 cell for the R7 cell fate determination is one of the best studied examples. Similarly, another example is demonstrated by the interaction of EGFr and *spitz* for the other R cells determination through Ras pathway and its downstream component. However, signal transduction cascades that involve only non-neural cell fate determination in the eye have not yet been illustrated. In the previous chapters, phenotypic and genetic characterization of the *rugose* (*rg*) gene showed that *rg* is required for cone cell determination. Identifying the cellular pathway through which *rg* interacts and performs its specific function for cone cell determination, may provide insights into the signal transduction mechanism that leads to non-neural cell specification. Since studies on genetic interactions are useful to identify components in a cellular pathway and uncover the relationships between these components. The study of genetic interactions of *rg* with other genes may define the pathway in which *rg* is involved. In this chapter, I present studies on the genetic interactions of *rugose* which suggest that *rg* interacts with components of

EGFr-mediated signal transduction pathway.

RESULTS

Identification of *rg* Modifiers

Dominant modifiers of *rg* were identified by screening for genes that directly or indirectly interact with *rg*. To identify genes that interact with *rg* we initially used a series of autosomal deficiencies (Bloomington stock center) and looked for dominant modifiers in a F1 screen. Homozygous *rg/rg* virgin females were crossed to males carrying an autosomal deficiency over a dominantly marked balancer. Non balancer F1 males which were hemizygous for *rg* and carried the deficiency over a wild type autosome were scored for the rough eye phenotype. We looked for either the suppression or the enhancement of the rough eye phenotype. Several known genes that play important roles in eye development and other signaling pathways were identified as *rg* modifiers (Table 10&11). The effects of a 50% reduction of the interacting gene and the effect of gain of function were studied. Interestingly, the results showed that *rg* interacts with components of the *Drosophila* EGFr (DER)-activated signal transduction pathway.

Table (10)

The genetic interactions of *rugose*

Interacting genes	alleles tested	*rugose* alleles tested	Effect on *rugose* rough eye phenotype
argos	*argos*^{257}	*rg*^{x6} , *rg*^{x10} , *rg*^{x7}	Suppression
	argos^{sty1}/*TM3*	*rg*^{x6} , *rg*^{x10}	Suppression
	argos^{sty2}/*TM6b*	*rg*^{x6} , *rg*^{x10}	Suppression
	hs argos	*rg*^{x6} , *rg*^{x10}	Enhancement
argos modifiers	*soba*^{sal}/*TM3*	*rg*^{x6} , *rg*^{x10}	Enhancement
	1040^{bulD}/*TM3*	*rg*^{x6} , *rg*^{x10}	Enhancement
	bul^{6D7}/*TM3*	*rg*^{x6} , *rg*^{x10}	Enhancement
Ras1	*sevd*^{2};*E(e.1B)th,st,c a/T21A*	*rg*^{1} , *rg*^{x6} , *rg*^{x10}	Enhancement
	sevd^{2};E(e.1B)/T21 A (*isogenic)*	*rg*^{1} , *rg*^{x6} , *rg*^{x10}	Enhancement
	Ras^{val12}	*rg*^{x6} , *rg*^{x10}	Suppression
rolled	*rl(1)*	*rg*^{x1} , *rg*^{x2} , *rg*^{x6} , *rg*^{x10}	Suppression

	lt rl stw	rg^{x1} , rg^{x2} , rg^{x10} , rg^{x8}	**Suppression**
Egfr	*Ellipse/CyO*	rg^{x6} , rg^{x10}	**Suppression (mild)**
	pn cn top[1] bw/CyO	rg^{x6} , rg^{x10}	**Enhancement**
	co stw pwn/CyO	rg^{x4}, rg^{x10}, rg^{x11}	**mild**
	2C82/CyO	rg^{x3}, rg^{x10}	**enhancement(?) mild enhancement**
rhomboid	**Df(3L)HR370*	rg^{x6} , rg^{x10}	**Suppression**
spitz	**Df(TW50)cn*	rg^{x6} , rg^{x10}	**Suppression**
Star	*S^{2}CyO/lm*	rg^{1} , rg^{x1} , rg^{x2} , rg^{x3}	**Enhancement**
	S^{54}/CyO	rg^{x4} , rg^{x5} , rg^{x6}	
	S^{11N23}/CyO	rg^{x7} , rg^{x8} , rg^{x9} , rg^{x10}	
	W1Siho/CyO	rg^{x11}	
	Shs8/CyO	rg^{x11}	**Suppression**
	cn bw/SM6a IKE 4.28.1	rg^{x6} , rg^{x10}	**Enhancement**
	cn bw/SM6a IV KE 2.4.2		"

cn bw/SM6a	***rg^{x6} , rg^{x10}***	"
VIII KE 4.1.1		
VII 30 El	***rg^{1} , rg^{x6} , rg^{x10}***	"
cn bw/SM6a		
II 2.27 E.1	***rg^{x6} , rg^{x10}***	"
cn bw/SM6a		
E3X12X	"	"
cn bw/SM6a		
cn bw/SM6a	"	"
VII KE.3.22		
cn bw/SM6a	"	"
II KE 1.0.4		
cn bw/SM6a	"	"
VIII KE 3.6.C		
II 2.8 EI	"	"
cn bw/SM6a		
cn bw/SM6a	"	"
IX KE 4.0.1		
E3R12X	"	"
cn bw/SM5		
E3R34X	"	"

	cn bw/SM5		
	E3RIIX	"	"
	cn bw/SM5		
Delta	*Dl*RF/*TM6c*	"	"
	*Dl(3R)DI*M2/*TM6c*	"	"
	*Dl*9B39 *es ca/TM6c*	"	"
	*ssDl*6B37e/*TM6c*	"	"
Suppressor of	*Su(H)/CyO*	rg^{x1} , rg^{x6}, rg^{x10}	**Suppression**
Hairless			

Example of Genetic Interactions of *rugose*

rg^{x6} + + + + $argos^{sty1}$

= = = X = = =

rg^{x6} + + + **TM3**

↓

rg^{x6} + $argos^{sty1}$

= = =

+ +

Double mutants (males) *rg*/Y; +/+; *argos*/+

Table (11)

Summary of the genetic interactions of *rugose*

Genes	Effect of 50% reduction in dosage on eye phenotype	Effect of hs-induced over expression/Gain of function allele etc
argos	Suppressor	Enhancer
Ras	Enhancer	Suppressor (Ras ^{val12})
rhomboid	Suppressor	Enhancer
Egfr	Enhancer	Suppressor (Egfr ^{Ellipse})
Star	Strong enhancer	Suppressor
Delta	Enhancer	Suppressor

rugose Interacts With Components of EGFr Pathway

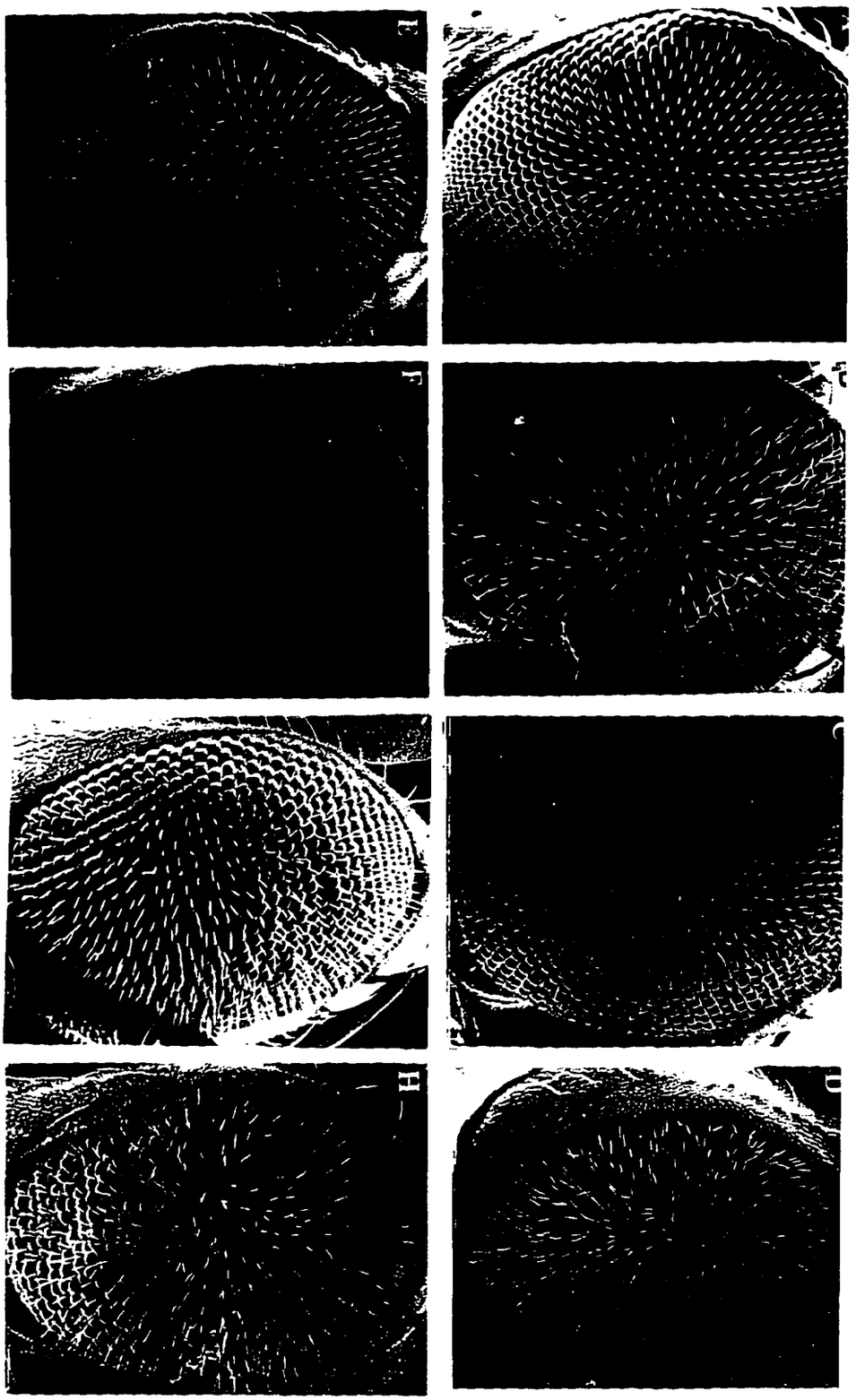
Ras1: ***Ras*1 is the *Drosophila* homolog of the vertebrate C-H-*ras*1 gene. It is a GTPase which acts as a regulator of RTK-activated signal transduction pathway. *Ras*1 is activated by the *Drosophila* homologue of the EGFr during oogenesis, eye development and wing formation (Simon et al., 1991). *Ras*1 mediated signal transduction cascade is required for all photoreceptors for proper differentiation (Zipursky and Rubin, 1994; Wassarman et al., 1996). One copy of the *ras* mutation enhances the *rg* mutant eye phenotype which suggests that *rg* may act upstream of *Ras* [Fig. 20&21C,D]. *Ras*^{val12}, a dominant constitutively active form of Ras, acts as a dominant suppressor of *rugose.***

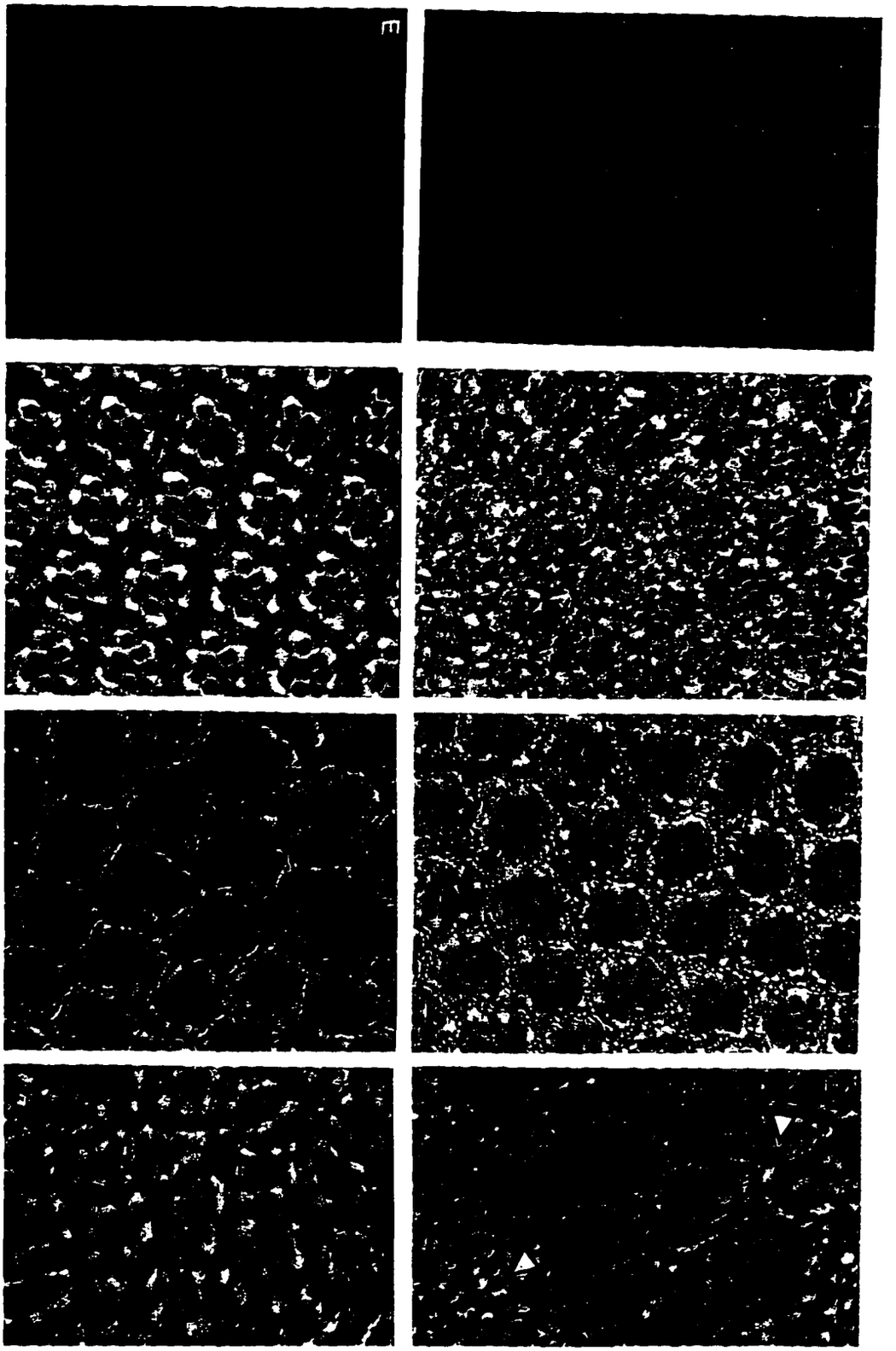
argos: ***argos*** **which also called** ***giant lens*** **and** ***strawberry*****, is a secreted protein that has an EGF motif. Argos acts non-autonomously and is required for the viability and cell fate determination in** ***Drosophila.*** **Mutations in** ***argos*** **result in the transformation of mystery cells (cells that have unknown fate in development) into extra R cells and leads to extra cone and pigment cell recruitment (Freeman et al., 1992).** ***argos*** **mutants also show extra wing veins and disrupt the lamina in the optic lobe (Wemmer and Klämbt, 1995). Overexpression of** ***argos*** **results in the reduction in the number of R cells, cone cells, pigment cells and shortening in the wing vein formation (Sawamoto et al., 1994).** ***argos*** **inhibits EGF-receptor function. It inhibits the activation of DER by** ***spitz*** **(Schweitzer et al.,1995). Mutations in** ***argos*** **strongly suppress the rough eye phenotype caused by** ***rugose.*** **In double mutants** ***rg/Y; argos/+*** **the rough eye phenotype is completely suppressed [Fig. 20&21E,F]. Heat shock promoter induced the expression of Argos gene product leads to enhancement of the** ***rg*** **mutant phenotype. Moreover, mutations that act as suppressors of** ***argos*** **such as** ***soba*** **and** ***bulge*** **function as strong enhancers of the** ***rg*** **eye phenotype [Fig. 20&21G,H].**

rolled (rl): ***rl*** **is also called** ***Sevenmaker*** **(*Sem*). It is a MAP kinase (mitogen-activated protein) which is activated by both** ***sevenless*** **receptor tyrosine kinase and the pathway activated by EGFr homolog.** ***rl*** **acts downstream of** ***Ras1*** **but upstream of the nuclear factor** ***sina*** **in the** ***sev*** **pathway.** ***rl*** **mutations affect signaling in the DER pathway and cause defects in wing venation (Brunner et al., 1994). A single dose of the** ***rolled*** **mutation suppresses** ***rg*** **rough eye [Fig. 23E,F]. This supports the notion that** ***rg***

Figure 20: Genetic interactions of *rugose*. Scanning electron micrographs of the compound eyes of A) Wild-type; B) $rg^{x\text{-}10}$; C) *ras*1/+; D) $rg^{x\text{-}10}$; *ras*1/+; E) $argos^{sty1}$; F) $rg^{x\text{-}10}$; $argos^{sty1}$/+; G) bul^{D}/+; H) $rg^{x\text{-}10}$; bul^{D}/+.

Figure 21: Anatomy of the double mutants. Light micrographs of the compound eyes sectioned tangential to the surface of the eye. A) Wild-type; B) $rg^{x\text{-}10}$; C) *ras*1/+; D) $rg^{x\text{-}10}$; *ras*1/+; E) $argos^{sty1}$; F) $rg^{x\text{-}10}$; $argos^{sty1}$/+; G) bul^{D}/+; H) $rg^{x\text{-}10}$; bul^{D}/+.





interacts with components of EGFr pathway.

DER (*Drosophila* homologue of the mammalian EGF receptor): DER is a receptor tyrosine kinase (RTK) which mediates signal transduction during the development of different tissues in *Drosophila*. DER function is required for the normal development of the retinular cells in the eye and the wing-vein formation (Xu and Rubin, 1993; Schweitzer et al, 1995). During eye development DER is required for both the proliferation of the imaginal disc cells and the differentiation of photoreceptors (Xu and Rubin, 1993). Mutations in the DER act as strong enhancers of the *rg* mutant phenotype. On the other hand, *Ellipse* (*Elp*), which is a dominant mutation in DER (Baker and Rubin, 1989), suppresses *rg* eye phenotype. These results suggest that *rg* may function in a DER-mediated signal transduction cascade.

The "Spitz" group: A group of genes called the "spitz group" plays important roles in the *Drosophila* developmental events. Mutations in these genes are lethal and affect structures derived from the embryonic ventral ectoderm. Interactions of *rg* with *Star, rhomboid,* and *spitz* demonstrate its relationship with members of the spitz group.

***Star (S)*: *Star* encodes a putative membrane protein and is expressed in photoreceptors R8, R2 and R5 (Kolodkin et al., 1994). Mutation at *S* locus results in abnormal eye phenotype. Heberlein and Rubin (1991), have shown that *S* is required in R8, R2 and R5 for normal determination and differentiation of the R cells, and that**

mutations in *S* act as enhancers of *rough* (*ro*) mutation. *Star* is also required for wing vein formation. *Star* interacts with mutations that mediate signaling by receptor tyrosine kinase such as *Ras1*, *Sos* and *E(sev)* (Heberlein et al., 1993). Heberlein and co-workers suggested that *Star* may be a part of a signal transduction cascade that activates *Ras* or respond to activated *Ras* in the R8, R2 and R5 precursors and that *Star* enhances the *Egfr* mutation. Studying the interaction of *rg* mutants with different alleles of *S* showed that *S* acts as a strong enhancer of the *rg* mutant eyes. In *Star/rugose* double mutant eye the retina is severely reduced and the lamina expands dramatically [Fig. 22]. Furthermore, overexpression of *Star* under the heatshock promoter suppresses the *rugose* rough eye phenotype [Fig. 22F]. These results suggest that *S* and *rg* may function in the same or a related pathway and are required for the normal development of the *Drosophila* eye.

<u>*rhomboid (rho)*</u>: *rho* is another member of the spitz group which encodes a putative transmembrane protein expressed in R8, R2 and R5 cells, and shows genetic interaction with EGFr gene (Freeman et al., 1992; Freeman, 1994). Ectopic expression of *rho* causes the non-neural mystery cells to adopt photoreceptors fate which indicated that *rho* plays a role in photoreceptor cell fate determination (Freeman et al., 1992). The expression of *rho* in the eye and the wing causes spitz/EGF receptor signaling. Freeman (1994) suggested that *rho* can act as a mediator between spitz/EGFr ligand receptor interaction. Our studies of *rho/rg* show that *rho* mutation acts as a dominant suppressor of *rg* mutant phenotype.

spitz (spi): *spitz*, one of the "spitz group" members, encodes TGFα homologue and produces a diffusible signal during eye development. *spitz* is required for photoreceptor cell determination and acts as a dominant suppressor of EGFr. Freeman (1994) suggested that Spitz protein may act as a diffusible ligand that triggers an EGFr-dependent cascade required for R cells determination. This activates Ras1 dependent signal transduction pathway. In our studies with *spitz*, it was found that *spi* acts as a weak suppressor of *rg* rough eye phenotype. This result supports the notion that *rg* may function in the DER mediated signal transduction pathway.

Interaction of *rugose* With Other Genes

Delta (Dl): *Dl* is a neurogenic gene which encodes the ligand in the *Dl-N* signal transduction cascade. Mutations in neurogenic genes results in expansion of the embryonic nervous system and reduction in the embryonic epidermis, in addition to the embryonic lethality. *Delta* and *Notch* are required for specification of R cells, cone cells, pigment cells and bristle precursors in the eye; and for the choice between epidermal and vein cell populations in the wing (Muskavitch, 1994; Parks et al., 1995). Mutation in *Dl* acts as a strong enhancer of the *rg* eye phenotype suggesting that *rg* may interact with components of neurogenic pathway [Fig. 23D,H].

Suppressor of Hairless [Su(H)]: *Su(H)* is the *Drosophila* homologue of the mammalian C promoter-binding factor 1 (CBF1) gene. This gene product is thought

Figure 22: ***Star*** **mutations act as strong enhancers of the** ***rg*** **phenotype. Scanning electron micrographs of A) Wild-type; B)** $rg^{x\text{-}6}$**; C)** S^{54}**; E)** $rg^{x\text{-}6}$**;**S^{54} **showing severe enhancement of the double mutant eyes compared with the wild-type and the mutant eyes. D) A transverse section in the double mutant eye showing the reduced size of the retina and the expanded lamina. F) Heat shock promoter induced overexpression of S+ protein suppresses** ***rg*** **phenotype.**

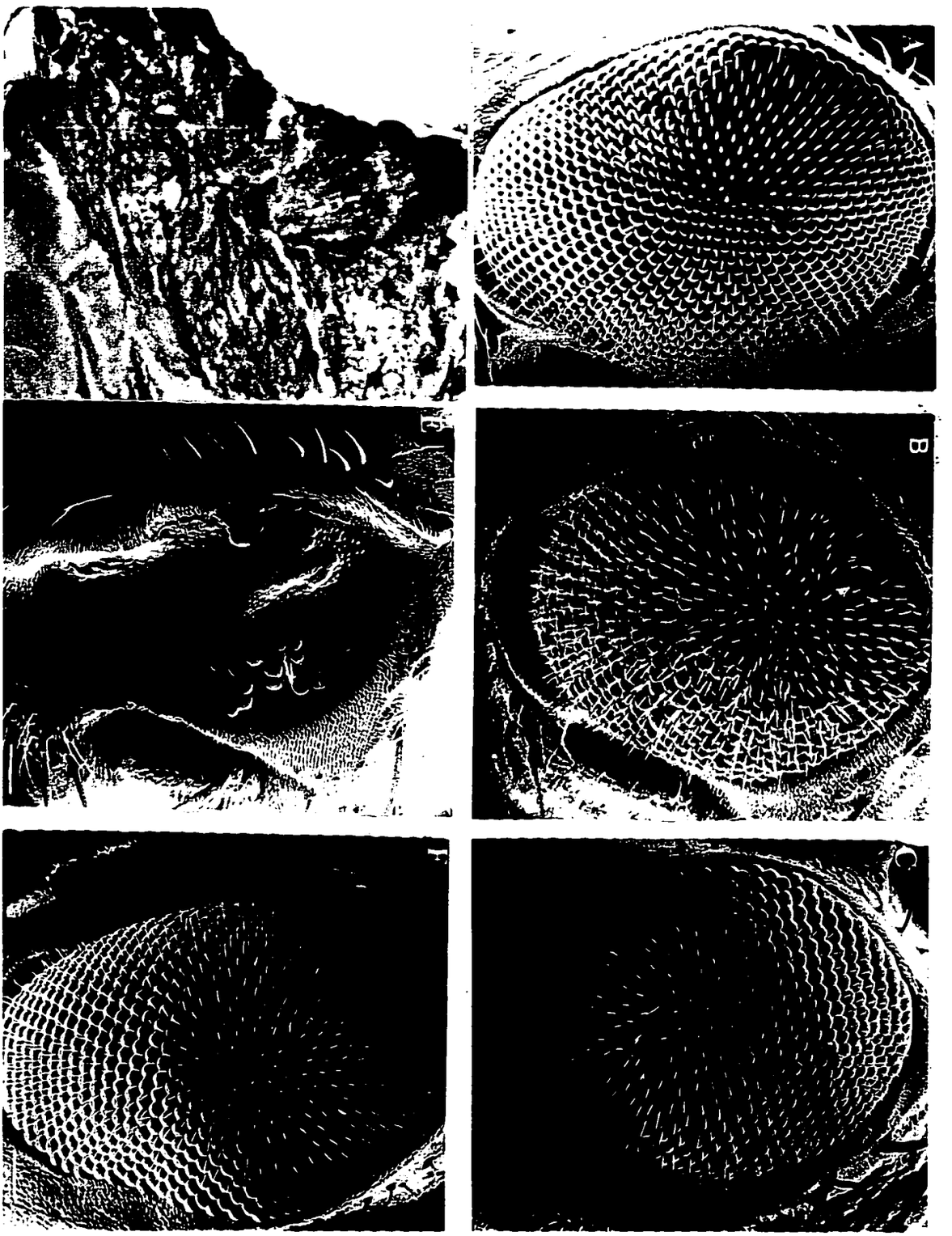
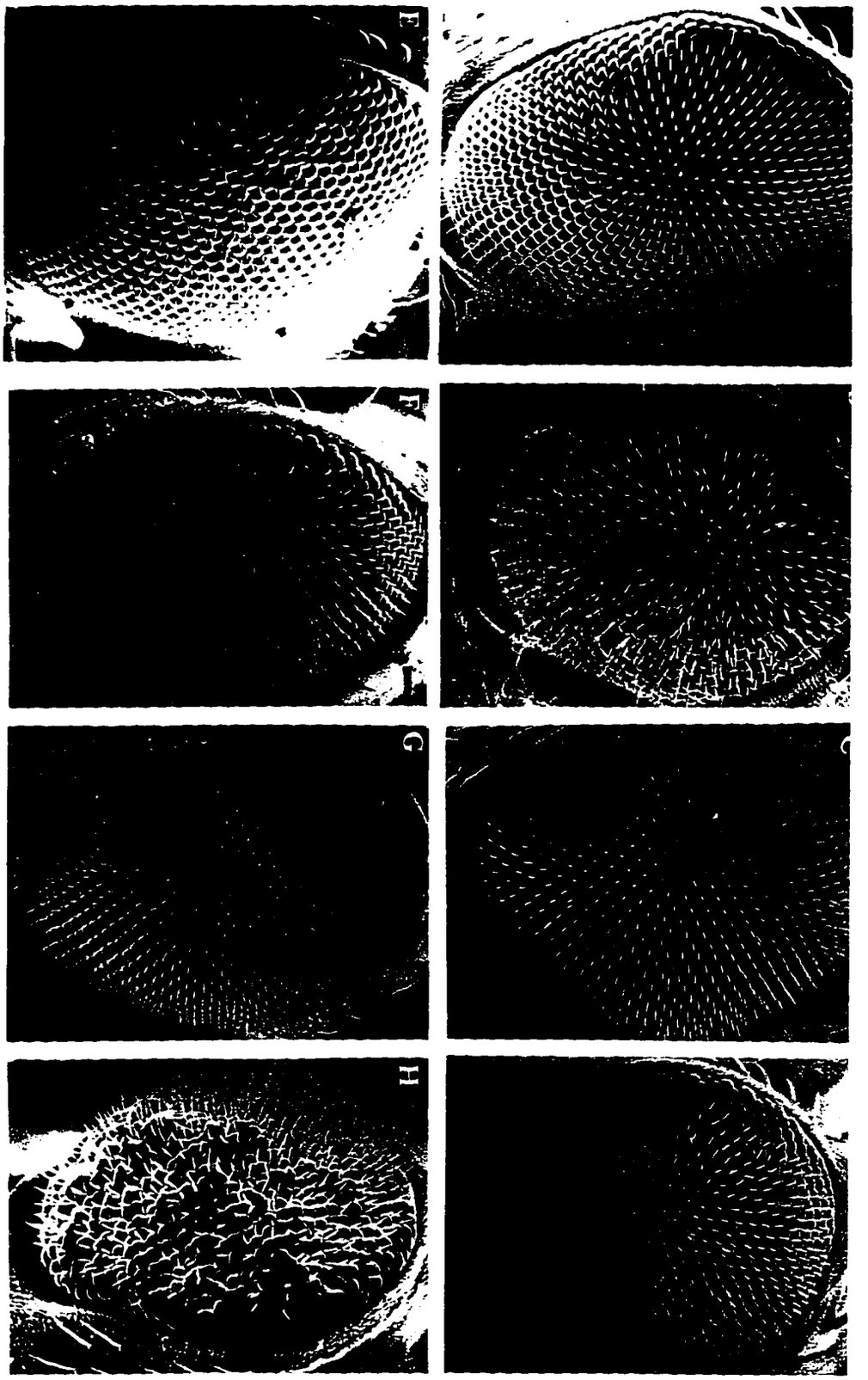


Figure 23: Genetic interactions of *rugose*. Scanning electron micrographs of double mutant compound eyes compared with wild-type and mutant eyes. A) Wild-type (CS); B) rg^{x-6}; C) *Su(H)*; D) *Dl*; E) rg^{x-6}; *rll*/+; F) *rll*/+; G) rg^{x-6}; *Su(H)*/+; H) rg^{x-6};*Dl*/+.



to mediate *Delta-Notch* signaling cascade and control cell fate choices (Fortini and Artavains-Tsakonas, 1994). *Su(H)* is required for the specification of sensory organ cells. Binding of *Notch* and its ligand *Delta* induces transportation of Su(H) into the nucleus (Fortini and Artavains-Tsakonas, 1994). A single copy of *Su(H)* suppresses the *rg* mutant phenotype which further supports the notion that *rg* interacts with components in *Delta-Notch* pathway [Fig. 23C,G].

DISCUSSION

Studies on genetic interactions have provided understanding of the functional role of a number of gene products and established a hierarchy of genes regulating cellular processes. In vitro experiment by Schweitzer and his colleagues (1995), has shown that *argos* acts as a negative regulator of the *Drosophila* homologue of the mammalian epidermal growth factor receptor (DER). They have proposed a model for *argos* function in which Argos competes with Spitz for binding to EGFr and blocking its signaling efficiency. In another model, they proposed that Argos binds to an unknown receptor and indirectly inhibits EGFr signaling. Our genetic interaction studies have shown that *rg;argos* double mutants have normal eyes. These results suggest that reducing *rg* level may lead to a reduced activation of DER, which is supported by the *rg* mutant phenotype that shows fewer or no cone cells in each

ommatidium. Since *argos* acts as a negative regulator of EGFr pathway, it may also act as a negative regulator of *rg*. Thus, reduced level of *argos* may compensate for the lack of *rg* protein function and elevate EGFr activation. This notion is supported by the finding that *argos* acts as a suppressor of *rg*. Wemmer and Klämbt (1995), have shown three genes, *bulge*, *soba* and *clown*, suppress *argos* eye phenotype in a dosage-dependent manner. They also showed that mutations in *clown*, *soba* and *argos* suppress *bulge* mutation. Therefore, these four interacting genes might function in a common pathway. Since *rg* showed interactions with *argos*, *soba* and *bulge*, it might also share a common pathway. Furthermore, hs-promoter driven overexpression of Argos acts as a strong enhancer of the *rg* phenotype suggesting that *rg* and *argos* may act in a common or a related pathway.

Activation of *Ras*1 is a key event in cell-cell interaction during the *Drosophila* eye development. Ras protein acts as a switch between the inactive GDP-bound form and the active GTP-bound form. This activity is controlled by two proteins that positively and negatively regulate Ras1 function (Gaul et al., 1992). It was found that activated Ras1 transforms nonneural cone cells into R7 cells in a dosage sensitive manner (Fortini et al., 1992; Karim et al., 1996). If EGFr-dependent Ras activity is critical for cone cell specification, the enhancement of *rg* eye phenotype by *ras* mutation suggests that *rg* may act upstream of *ras* as an effector of cone cell development through EGFr Ras-dependent pathway. Our results which show that Ras^{val12}, a consistively active *Ras* mutation, acts as a dominant suppressor of *rg*, is consistant with this idea. Recent studies have shown that in Ras^{val12} mutants, there is an increase in

cone cell number further supporting the idea of the involvement of *Ras* in cone cell specification.

Parks and co-workers (1995), demonstrated that *Delta* function is required for correct specification of cone cells and pigment cell lattice. They have shown cone cell multiplication due to reduction in the *Dl* function near the morphogenetic furrow which indicates that *Dl* is required for cone cell fate choice. During pupal development, vein cells differentiate into compact pigmented cells that lack adhesion to the wing surface resulting in the formation of the wing veins. Reductions in *Dl* or *Notch* function lead to the formation of thick veins due to the over recruitment of vein cells, and to the reduction in the wing blade epidermal cells (Muskavitch, 1994). This finding indicates that *Dl* and *N* functions are essential for the normal number of cells that adopt the vein cell fate. When *Dl* and *N* signaling is increased than normal many cells adopt the epidermal cell fate result in vein shortening. The pleiotropic phenotypes of *Dl* and *rg* in the eye pattern formation and wing vein specification strongly suggest their interaction.

Heberlein and co-authors (1993), have reported that *Star (S)* interacts with *ras* in a dosage sensitive manner. The precise mechanism of this interaction is not clearly understood. They also suggested that *S* is required for the formation of the wing veins. Kolodkin et al.(1994), showed genetic interaction of *S* with *sevenless* and *EGFr* indicating an early role of *S* in the eye development through Sevenless-EGFr-Ras-mediated signal transduction pathway. Freeman (1994), has determined the role of *spitz* in R cell differentiation, and reported that *rhomboid* acts as a mediator of Spitz-

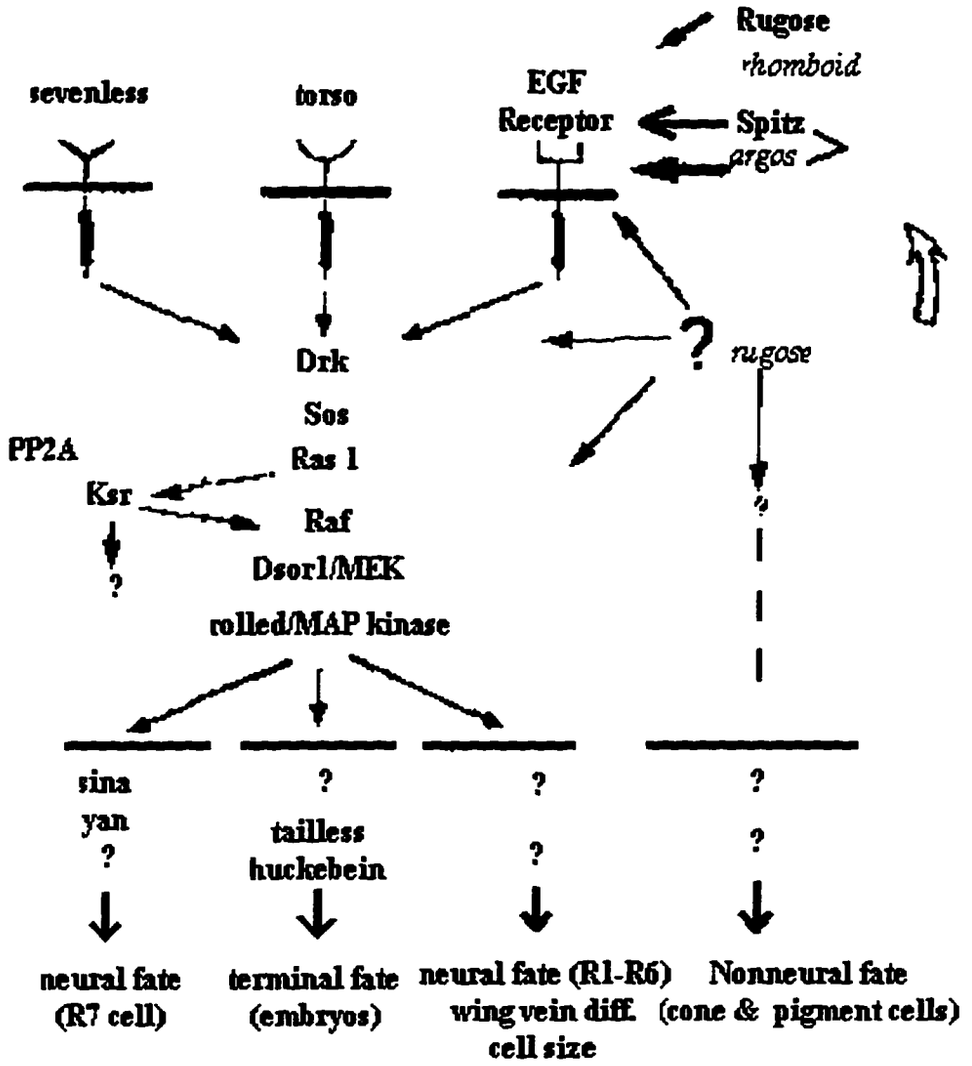


Figure 24: Diagram showing the components of three different receptor tyrosine kinase signaling pathways (fig. adapted from Brunner et al., 1994 and modified). Genetic interactions suggest that Rugose may participate in the signaling cascade activated by the EGF receptor. The three receptors: Sevenless, Torso and EGFr utilize a similar signaling cascade and share several down stream components. The arrangement of the components and the inferred interactions in the cascade is based on genetic epistasis experiments. Genetic epistasis suggest that Rugose may function as a modulator upstream of Ras. It is possible that Rg functions in a parallel pathway specific to cone cells.

EGFr interaction in the eye development. Freeman has suggested that R cells other than R7 use Spitz/EGFr activated Ras1 signaling for their determination and that *S* acts in the same pathway. In *rhomboid* mutants L2-L5 veins do not reach the wing margin while the cross veins are normal. It was found that *rho* and *argos* interact and double mutations enhance the wing phenotype and eliminate the longitudinal veins completely which suggests that they may function in a common pathway specific for wing vein development (Sawamoto et al., 1994). *rg* wing phenotype as well as its embryonic lethality, and the interaction with members of the spitz group suggest their function in a similar or a related pathway.

Experiments from several laboratories have shown that activation of DER results in the onset of a signal transduction cascade involving Ras and MAP kinases. The present study demonstrated that *rg* interacts with components of EGFr (DER)-activated signal transduction pathway which indicates that *rg* is involved in DER pathway and shares downstream components. It is more likely that *rg* functions as a mediator of DER specific to cone cell determination. Probably, *rg* plays in cone cell specification a role similar to *spitz* in R cell development. Based on the phenotypic analysis and the genetic interactions of *rg* which I have uncovered, several models for the cellular function of *rugose* can be formulated. Among the simplest of these, I propose that Rugose may be an effector of cone cell development exerting its influence through the EGF receptor (DER) [Fig. 24]. The interaction of Rg and DER could be either direct or indirect. In this model, Rg positively activates DER in the cone cell precursors and initiates or promotes the cell determination pathway. This model

predicts that: 1) reducing Rg levels would lead to a reduced activation of DER. This is supported by the result that mutations in *rg* result in fewer cone cells; 2) a negative regulator of DER, such as Argos, would also be a negative regulator of Rg. Thus, reduced levels of Argos would be expected to compensate for the lack of *rg* function and would elevate DER activation. This prediction is supported by the experimental observation that *argos* mutations act as suppressors of *rg*; 3) overproduction or elevated levels of Argos would be expected to have the opposite effect. The results showing that heat shock promoter-driven Argos over-expression results in enhancement of the *rg* phenotype (presumably due to reducing DER activation) support this prediction; 4) over-expression or ubiquitous expression of Rg would result in hyperactivation of the DER pathway and lead to gain-of-function DER mutant phenotypes in the eye and wing. A consequence of this would be the overproduction of cone cells, R cells and wing veins. This should be readily testable once the *rg* gene is cloned and characterized. At this point the proposed model for the cellular function of Rg may be simplistic and the cellular role of *rg* could be far more complicated. However, the important feature of the model is that it offers several predictions that can be tested experimentally. Taken together, *rg* interactions and the wing phenotypes that are characteristic of mutations in the EGFr pathway, indicate that *rg* may either function through EGFr pathway or via a novel/parallel pathway regulating cone cell determination. These approaches are based solely on the genetic interactions and the phenotypic analysis of *rugose* mutations. Molecular studies are required to provide insights into the nature of *rg* gene product and to facilitate the understanding of the *rg*

function in the *Drosophila* development. Towards these aims, the next chapter provides some molecular analysis of the *rg* gene and attempts of *rg* gene cloning.

METHODS

In order to uncover dominant interactions in a F1 screen, virgin *rg/rg* females were mated to males carrying known autosomal deficiencies over a balancer (obtained from Bloomington stock center). First generation resulted from these crosses were screened for non-balancer male modifiers that enhance or suppress the *rg* rough eye. The known break points of these modifiers were used to determine any well known genes within the deficiencies by searching the fly data base. Deficiency stocks were out crossed to minimize the genetic noise and background effects. Homozygous or hemizygous *rg* mutants were crossed with mutations in the defined genes. For each mutation, multiple alleles of *rg* and the interacting mutations were tested.

CHAPTER V

MOLECULAR ANALYSIS OF THE *rugose* GENE

INTRODUCTION

Molecular analysis of a gene is important in understanding the biochemical nature of the gene product and the cellular mechanism in which the gene functions. Towards this aim, localization and molecular cloning of the gene of interest are the important first steps. To understand the molecular organization of the *rg* locus and the biochemical nature of its protein product, I have initiated a molecular analysis of *rg*. In this chapter, I present preliminary steps in molecular cloning of *rg*. In *Drosophila*, several strategies can be used to clone genes of interest. Prior knowledge of the genetic locus determines the strategies that can be applied for cloning. These techniques depend on the identification of the cytogenetic location of the gene and the availability of suitable probes to clone the gene. Probes from previously cloned sites that are located near to the gene in question can be used to initiate chromosomal walking. Another powerful technique for molecular cloning is transposon tagging when a transposable element is inserted in or near a gene. The transposable element can be used as a probe to recover the flanking DNA sequences at the insertion site. The chromosomal location of *rg* was determined by mapping using stocks carrying deficiencies and duplications. Initially, a transposon tagging technique was selected.

This choice was based on the data that a number of P-transposon induced *rg* alleles were isolated and several of these were found to be insertions by reversion analysis. In our hybrid-dysgenic crosses we mobilized the P-Lac-w P-element (Bier et l., 1989). This P-element carries the Lac-Z gene along with the antibiotic markers and the bacterial origin of replication. Thus, the P-element insertion permits plasmid rescue method for the isolation of the flanking DNA sequences from the insertion site. Although in my experiments the initial steps of plasmid rescue cloning were successful, the subsequent steps failed to yield convincing and consistent results. As such, this approach was abandoned. As an alternative, a chromosomal walk was initiated by using a DNA probe derived from the deadhead locus. A putative *rg* genomic DNA clone (RGD1) has been isolated. This putative clone of *rg* was used as a probe to isolate *rg* cDNA clones from an eye-disc cDNA library. The data from these studies are presented in this chapter. These serve as an important first step in the molecular characterization of *rugose*.

RESULTS

Chromosomal Localization and Fine Mapping of *rugose*

A. Deficiency Mapping

Previous genetic mapping by Salz (1992), has shown that *rg* is located in region 4 on the X-chromosome distal to *sansfille* (*snf*) and *deadhead (dhd)* genes. Deficiency

mapping of *rg* gene locus was accomplished by using a series of overlapping X-chromosome deficiencies with identified breakpoints. In these studies, rg^{x6} males were crossed to virgin females that carried the deficiency on one X-chromosome and a balancer on the other. F1 females were screened for rough eye phenotype. In each of the crosses, rough-eyed nonbalancer heterozygous females were scored. These results determined that the tested deficiencies uncovered the recessive *rg* mutation. The cytological position of the *rg* gene locus was narrowed to the region between 4E and 4F on the X-chromosome [Fig. 25].

B. Mapping with a Duplication

As a result of the deficiency mapping studies, the *rg* locus was determined at the cytological position 4E-4F. Therefore, flies that carry a duplication of the 4E-4F region on the second chromosome were used to test whether the duplication covers the recessive *rg* mutation. The eye phenotype of F1 males that resulted from crossing rg^{x6} males to ywf,xx; ywsnf^{210}; RDUP(W^+)/+ females were indistinguishable from the wild-type. The results indicated that rg^{x6} is covered by the duplication and that *rg* maps within that chromosome region further supporting the previous deficiency mapping results.

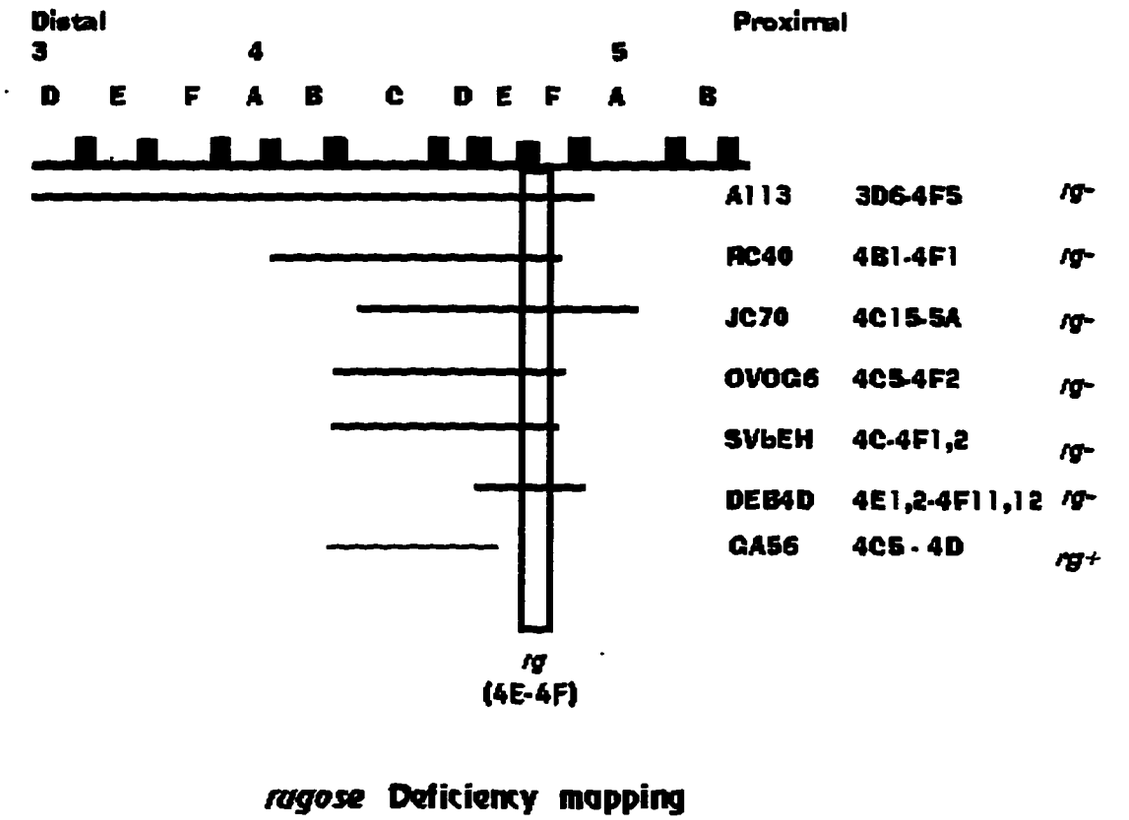


Figure 25: Deficiency map of the *rugose* locus. The cytological position of the *rg* locus on the X-chromosome was determined by using 7 separate deficiencies that have previously identified breakpoints. The *rugose* locus was detected in 4E-4F region.

Plasmid Rescue and Attempts To Clone The *rugose* Locus

The P-element used to induce mutations in the *rg* locus was mobilized in a hybrid dysgenic cross. w*dhd*^{p8}/FM7 males which carried a single P-lacZ vector in the *deadhead* (*dhd*) gene (Salz et al., 1994), were crossed to M' cytotype females that carry a source of transposase, Δ2-3. The P-Lacw vector, includes fusion gene that contains

a bacterial origin of replication and the β-lactamase gene coding for ampicillin resistance (Bier et al., 1989). In addition this vector has unique restriction sites that allow the isolation of the genomic sequences flanking the P-insertion. Therefore, the rescued plasmid should contain DNA from the vector and the fly's genome. Genomic DNA was isolated from rg^{p2}, rg^{p5} and the wild-type (Canton S), circularized and used for plasmid rescue high efficiency transformation. Plasmid rescue selected rg^{p2} colonies were isolated separately and plasmid-DNA with the genomic DNAs was isolated and recovered from each transforment. The flanking sequences of the mutant genomic DNA was used to probe genomic southern blots. No reproducible hybridization was observed after several trials. Hence this approach has aborted and *rg* cloning was initiated by a chromosomal walking.

Isolation of A Putative *rg* Genomic DNA Clone By Chromosomal Walking

The *rg* gene is located in a well characterized area on the X-chromosome close to previously cloned genes. As *rg* was mapped to a region distal to *dhd* gene, a 2.5Kb genomic Bam-Kpn fragment from the *dhd* locus was used as a probe to initiate a chromosomal walking. As a control we checked the *rg* mutant alleles for any restriction fragment polymorphisms (RFLPs) in the *dhd* region. To do this, the *dhd* probe was hybridized on southern blots of restriction digests of genomic DNA from wild-type (parental) and *rg* mutant alleles. The *dhd* fragment showed hybridization to a single 5.8Kb band in the wild-type and *rg* mutants genomic DNA [Fig. 26]. The results show

that there were no DNA polymorphisms at the gene locus in *rg* mutants. This suggests that no correlation exists between the molecular organization of the *dhd* clone and the rough-eye phenotype caused by the *rugose* mutation. The *Drosophila* genome project has available *Drosophila* genomic DNA clones in a P1 phage vector. These vectors carry between 80-100Kb genomic DNA inserts and have convenient restriction enzyme sites for chromosomal walking. We obtained the P1 clones which contained the genomic DNA from the 4E-4F region. Eight relevant P1 clones from 4E-4F region were obtained from this collection. To identify the P1 clone(s) that includes the *rg* gene region, a southern blot from different P1 clone genomic DNAs was probed with the 2.5Kb fragment from *dhd*. This probe showed hybridization in two separate P1 clones, 82-63 and 5-14 which indicates that the two clones included the region of interest [Fig. 27]. Thus, 82-63 and 5-14 are relevant candidates to initiate a chromosomal walk within the P1 genomic DNA. One of the two P1 clones was selected for further analysis. The criteria used was the following; I used the entire P1 clone as a probe in a genomic southern blot of parental (wild-type) and *rg* mutant alleles for each P1 clone. The entire clone DNA along with the vector sequences was digested with EcoR1 and labeled by random hexamer labeling and used as a probe on a southern blot. Of the two, only the clone 82-63 detected RFLPs, suggesting that the DNA in the P1 clone may be derived from the *rg* region. Thus the P1 clone 82-63 was selected for further analysis.

Figure 26: Southern blot analysis of *rugose* mutants. Genomic DNA from the wild-type (CS) and thirteen *rg* mutant lines was digested with EcoR1 and probed with 2.5Kb BamHI-KpnI fragment derived from a *dhd* genomic clone. The banding pattern of all mutant lines is similar to the wild-type. Lane (1) CS, (2) rg^{p2}, (3) rg^{p5}, (4) rg^{x1}, (5) rg^{x2}, (6) rg^{x3}, (7) rg^{x4}, (8) rg^{x5}, (9) rg^{x6}, (10) rg^{x7}, (11) rg^{x8}, (12) rg^{x9}, (13) rg^{x10}, (14) rg^{x11}. Please note the apparent mobility differences is due to an electrophoresis artifact does not represent actual DNA polymorphisms.

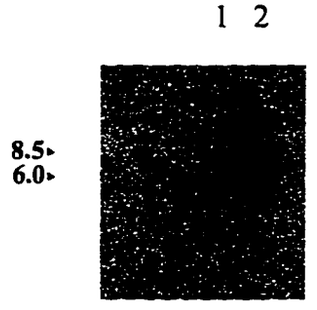


Figure 27: Southern blot analysis of P1 clones. Genomic DNA from P1 clones was digested with EcoR1 and probed with 2.5Kb BamHI-KpnI fragment derived from *dhd*. Two P1 clones (1) 82-63 and (2) 5-14 showed hybridization with the probe.

Identification and Isolation of A Putative *rg* Genomic DNA Fragment

The P1 clone 82-63 contains about 80 Kb of the *Drosophila* genomic DNA as an insert. Initially I mapped the DNA fragments which hybridized to the 2.5 Bam-Kpn *dhd* probe on southern blots. Three fragments were detected. Of the three EcoR1 fragments only one, a 4.8Kb fragment detected RFLPs on southern blots containing wild-type parental, *rg* mutants and revertant genomic DNA. This 4.8Kb EcoR1 fragment was designated RGD1 and used for further analysis.

Southern Blot Analysis

Genomic DNA from different *rg* alleles and revertants were isolated and used to prepare southern blots. The P1 clone DNA sequences that flank the *dhd* fragment were isolated and used to probe southern blots from *rg* mutants and revertants. One of the flanking sequences showed hybridization to *rg* mutants genomic DNAs. The putative *rg* genomic DNA clone was isolated from the P1 genomic clone (82-63) and named RGD1. Restriction fragment polymorphisms (RFLPs) were observed in different *rg* mutants DNA which determined that the isolated DNA clone was actually derived from the *rg* locus [Fig. 28]. Molecular characterization of the *rg* gene has been initiated by using the isolated putative *rg* clone. RGD1, which is about 4.8Kb DNA EcoR1 fragment, was subcloned in pBs phagemid vector (Stratagene).

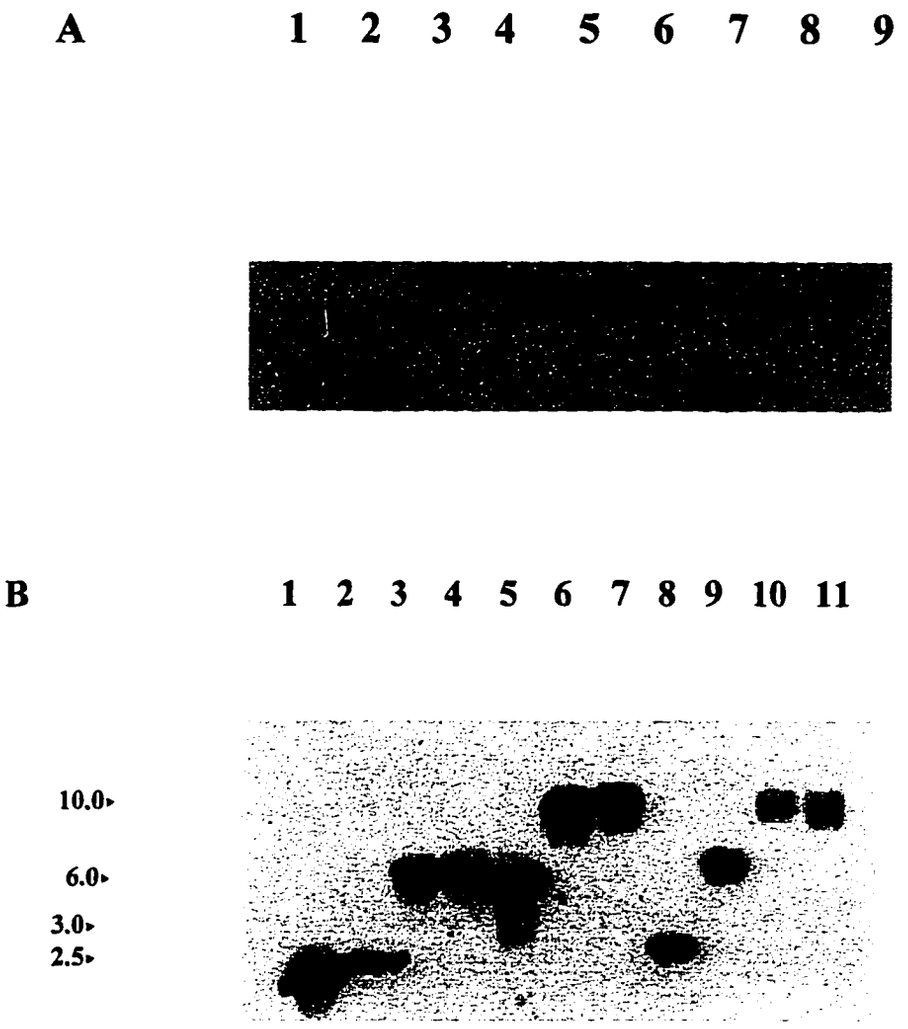


Figure 28: Southern blot analysis of *rugose* mutants: The putative RGDI clone (4.8Kb EcoR1-EcoR1 fragment) was used to study the organization of this locus in the *rugose* mutants. Blots probed with RGDI showed restriction fragment polymorphisms with *rg* mutants. [A] A southern blot from (1) CS, (2) dhd^{p8}, (3) rg^{p2}, (4) rg^{p5}, (5) rg^{x1}, (6) rg^{x2}, (7) rg^{x3}, (8) rg^{x4}, and (9) rg^{x5}. [B] A southern blot from (1) CS, (2) dhd^{p8}, (3) rg^{p6}, (4) rg^{p9}, (5) rg^{1}, (6) rg^{x6}, (7) rg^{x7}, (8) rg^{x8}, (9) rg^{x9}, (10) rg^{x10}, (11) rg^{x11}.

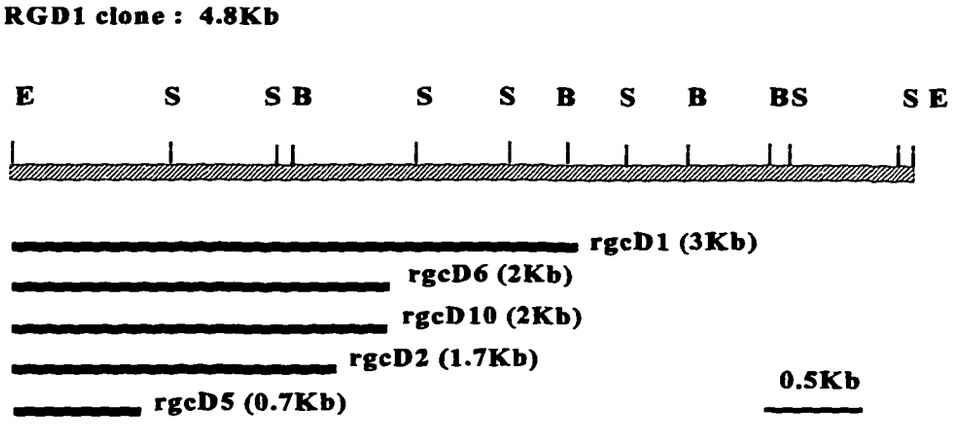


Figure 29: Map of the RGD1 clone. RGD1 EcoRI(E) clone was mapped with enzymes Bam HI (B) and Sau 3A (S). Five rgcDNA clones were isolated from *Drosophila* eye disc cDNA library.

Restriction Map of RGDI

Restriction endonucleases and southern blot analysis were used to determine the positions of the restriction enzyme recognition sites and a restriction map was constructed [Fig. 29].

Isolation of *rugose* cDNA

Isolation of the cDNA clones from the *rg* locus is an important step towards the characterization of the *rg* protein. In order to isolate the wild-type cDNA(s) from the *rg* locus, the 4.8Kb EcoR1 fragment (RGDI) was random hexamer labeled and used to probe cDNA libraries. Five cDNA clones were isolated from a *Drosophila* eye disc

cDNA library by screening 500,000 λgt10 phages. The isolated cDNA clones were named rgcD1 (3Kb), rgcD2 (1.7Kb), rgcD5 (.7Kb), rgcD6 (2Kb) and rgcD10 (2Kb). The *Drosophila* Rosy head 3-8 library was also screened by using RGDI as a probe. No cDNA clone was isolated by screening 590,000 phages.

DISCUSSION

In order to obtain P-element-induced mutants, a stock that already contained a P-element (*dhd*/FM7) cytologically close to *rg* map position, was used. The mobilization of this P-element induced mutations in the *rg* gene. Remobilization and reversion analysis in chapter 2 (pages 21 & 22) have shown that the obtained mutations (rg^{p2} and rg^{p5}) were due to insertions in the *rg* gene locus. Thus, using plasmid rescue protocol to initiate *rg* cloning was a first choice. Unfortunately, this initial attempt was unsuccessful. The data obtained from the chromosomal walking within a P1 clone from *Drosophila* genomic DNA was more successful. The isolation of the *rg* clone, RGDI, that showed restriction fragment length polymorphisms (RFLPs) in southern blots, indicates that RGD1 is derived from the *rg* locus. This RGD1 clone facilitated the isolation of *rg* cDNAs clones which suggests that it includes coding regions of the *rg* locus. This would facilitate further experiments on the molecular cloning. Cone cells, in addition to secreting the lens are important for the retinal pattern formation. Studying a gene such as *rugose* may lead to a better understanding of the cone cell

specific function in the eye. It is my hope that the data presented in this study will initiate and be used to facilitate future researches in *rg* molecular analysis and its protein characterization.

MATERIALS AND METHODS

Deficiency Mapping

Complementation studies were designed to localize *rg* gene in the X-chromosome. Virgin females that carry deficiencies on the X-chromosome were crossed to rg^{x6} males. Seven different deficiencies were used, Df(1)RC40/FM7, Df(1)JC70/FM7c, Df(1)A113/C(1)Dx,ywf, Df(1)OVOG6/FM7, $Df(1)SVb^{EH}$/Blmsc, Df(1)GA56/FM7 and Df(1)ywDEB4D/FM7. These deficiencies have identified breakpoints mapped to 4B1-4F1, 4C15-5A, 3D6-4F5, 4C5-4F2, 4C-4F1,2 and 4E1,2-4F11,12 regions respectively (Banga et al., 1986 and Oliver et al., 1988). ywf,XX,$ywsnf^{210}$; RDUP(w+)/+ flies that carry a duplication which covers 4C11,12 - 4F11,12 interval of the X-chromosome on the second chromosome were crossed to rg^{x6} mutants. F1 males were scored for smooth eyes to determine if the duplication covers *rugose* mutation.

Plasmid Rescue

Rapid small scale isolation of the flies genomic DNA and plasmid rescue from LacZ lines were done according to the method book from Dr.Rubin lab (1990). High efficiency transformation protocol was used as described by Hanahan (1985). Plasmid DNAs were isolated by the minipreps alkaline lysis method as in Maniatis et al. (1982).

Southern Blot Analysis

***Drosophila* genomic DNAs were prepared as described in Rubin's method book (1990). DNA digested with the appropriate restriction endonucleases and was electrophoresis on 0.8% agarose gels with 1μg/ml ethedium bromide in 0.5X TBE and run at 50V. Southern blots were prepared according to standard methods described by Maniatis et al (1987). Probes were labeled with ^{32}P using random-hexamer DNA labeling kit from Boehringer Mannheim. The *deadhead* clone, 2.5Kb BamHI-KpnI fragment mapped into 4F1,2, was donated by Dr. H. Salz.**

RGD1 Cloning

Searching the flybase the following P1 clones were obtained and used to prepare a southern blot. The isolated clone (RGDI) was subcloned in pBS(+/-) phagemids vector from Stratagene and transformed into XL-1Blue MRF' host cells following the procedure described by Stratagene.

P1 Name	Locn	Region (from-to)
DS02520	27-24	4D3 - 4E1
DS00457	05-73	4E1 - 4E2
DS07839	82-63*	4E1 - 4E2
DS00398	05-14*	4F1 - 4F3
DS05631	59-63	4F2 - 4F3
DS09071	95-47	4F2 - 4F3
DS00572	06-92	4F11 - 5A1
DS04273	45-49	4F11 - 5A1

*P1 clones that showed hybridization with the *deadhead* fragment.

Isolation of cDNA Clones

Eye disc and Rosy head (3-8) cDNA libraries in λgt10 vector were gifts from Dr. G. Rubin lab, U. C. Berkeley. cDNA libraries were kept in 2% DMSO and stored at -20°C. Plating bacteriophage λ and immobilization of plaques on nitrocellulose filters were done according to Maniatis et al. (1987) which was modified from Benton and Davis (1977). Hybridization to nitrocellulose filters, picking bacteriophage plaques and preparing plate lysate stocks were followed the standard method described by Maniatis et al. (1987).

CHAPTER VI

SUMMARY & CONCLUSIONS

The development of the compound eye, in the *Drosophila melanogaster*, is one of the best characterized systems to study cellular pattern formation and genetic interactions. In this system cell-cell communication is necessary for the initiation and maintenance of developmental decisions. One approach, is to obtain genetic variants in which defects can disrupt developmental events leading to abnormal phenotypes. The *Drosophila* compound eye consists of about 800 unit-eyes called ommatidia, which are arranged in a regular symmetrical array giving the eye a characteristic smooth appearance. Each unit eye or ommatidium includes eight photoreceptor cells (R cells), four cone cells, six pigment cells and a mechanosensory group. Any mutation that perturbs the structure and organization of the eye is readily distinguished by the irregular rough morphology. *rugose* (*rg*) is one of several genes that are required for the maintenance of the smooth pattern of the eye. During development, both neural and nonneural cell determination requires proper cell-cell communications and positional cues. These signals play critical roles by inducing adjacent cells to differentiate. Several studies have clearly established that, in the eye, cells differentiate by sequential inductions and cell-cell interactions and that cell lineage relationships have no critical role in specifying cell fates. Although a number of studies have

addressed the mechanisms involved the neural cell determination, nonneural cells did not gain much attention. In the present study, mutations in the *rugose* gene disrupted the regular arrangement of the eye due to defects in nonneural cells. *rugose* mutants showed fewer lens-secreting cone cells and abnormal arrangement of the pigment cell lattice. Moreover, *rugose* mutants developed abnormal fenestrated membrane that failed to support the inner surface of the retina. Some photoreceptor cells that lost the support fell beneath the retina into the lamina. *rugose* mutants also showed defective wings which indicates that *rg* plays a role in the cellular determination mechanism that leads to wing formation. Genetic studies of *rugose* showed that *rg* interacts with components of the EGFr pathway. These data suggested that *rg* is involved in cone cell fate determination through EGFr pathway or a related pathway for the proper ommatidial assembly and normal wing development. Although the isolated lines of *rugose* are viable, several alleles exhibited a semi-lethal phenotype suggesting some early embryonic defects and a more general role of *rg* gene in the *Drosophila* development. In addition, the first steps towards *rg* cloning and its molecular characterization were encouraging. A putative *rugose* genomic clone (RGD1) has been isolated. *rugose* cDNAs were also isolated by screening an eye disc cDNA library. Taken together, data from *rg* phenotypic analysis and genetic interaction studies have made this gene an interesting element for further studies. Characterization of the *rg* gene and its protein product is a subject which requires future investigation for better understanding of nonneural cell fate determination. Future studies are required to determine the DNA sequence of the *rg* cDNA and the corresponding genomic DNA

regions encoding them. Germline transformation will define the physical limits of the *rg* gene. The pattern of *rg* expression could be determined by studying the temporal and spatial patterns of *rg* mRNA expression. Somatic mosaic analysis should be performed in order to determine the cell and tissue requirement of *rg* function. This will determine whether *rg* function is autonomous or non-autonomous to the cone cells. These results will be important in understanding the role of *rg* in cone cell determination.

Studies on *rg* may uncover a new pathway which is specific for cone cell determination. On the other hand, studies on *rg* may reveal new components of the RTK signaling cascade responsible for non-neural cell determination. Thus, studies presented here and future experiments will contribute significantly in understanding cellular pattern formation and the role of cellular signaling pathways.

In Conclusion:

1. *rugose* gene product is required for normal differentiation of the cone cells in the *Drosophila* compound eye. The defects in cone cell differentiation are evident at the earliest stages suggesting that *rg* function may be required for the proper specification of the cone cells.

2. Mutations at the *rugose* locus result in abnormal lattice and fenestrated retinal basement membrane development as a consequence of the aberrant cone cell specification.

3. *rugose* function is necessary for normal wing development. This wing phenotype seen in *rg* mutants is a characteristic feature associated with mutations in the EGF receptor (DER) signaling pathway.

4. Genetic interactions of *rugose* with components of the EGF receptor mediated signal transduction pathway and participate in cone cell specification suggest that *rg* may function in the EGFr pathway or a related pathway.

Appendix I

In press J. Neurobiology

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

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RUNNING TITLE: Photoreceptor differentiation in *Drosophila*

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SUMMARY

The compound eye of *Drosophila* is a reiterated pattern of eight hundred 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. Our previous work has shown 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. In this study we have analyzed the R cell composition of both developing as well as the adult eyes of *rap* mutants employing a variety of R-cell specific markers. We show that, in *rap* mutants while 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.

Key words: Photoreceptors, neural development, retina, cell-cell interaction, *Drosophila*, eye development, *rap*

Appendix II

Table (12)

Drosophila stocks used in the study

Strain	Genotype	Source
argos	*argos*^{sty1}/TM3	Dr. Okano (University of Tsukuba, Japan)
	argos^{257}	"
	hs argos#4 (Transgenic)	"
	argos^{sty2}/*TM6b*	"
argos modifiers	*soba*^{sal}/*TM3*	Dr. Klambt (University of K ln)
	bul^{6d7}/*TM6*	"
	1040^{bulD}/TM3	"
Ras 1	*sevd 2;E(e.1.B)th st ca/ T21A*	Dr. Simon (Stanford University)

	sevd-2;E(e.1.B)/T21A (isogenic)	"
Delta	***Df(3R)DfRF/TM6c***	**Dr. Muskavitch (Indiana University Bloomington)**
	Df(3R)DfM2/TM6c	"
	ssDf(3)B37e/TM6c	"
	ru h th st cu sr, Df(3)B39esca/TM6c	
rolled	***lt[1]***	**Bloomington stock center**
	rl[1]	"
deadhead	***w dhd ^{p8}/FM7***	**Dr. Hellen Salz (Case Western Reserve University)**
Suppressor of Hairless	***Su(H)(1)In(2L)Cy,In(2R)Cy ,Cy[1]pr[1]***	**Bloomington stock center**
	w/w; 2-3SbTM3/Drop	**Dr. Hellen Salz**

	*ywf,XX;ywsnf*210*;RDUP*	"
	(w+)/+	
	Df(1)A113/C(1)DX,ywf;Dp(	**Bloomington stock center**
	1;2)w[=64b]/+	
	Df(1)JC70/FM7c,sn[+]	"
Egfr(torpedo)	*pr cn top*1 *bw/CyO*	**Dr. Govind, City College of NY**
	Ellipse/CyO	**Indiana stock center**
Star	S^{2}*/CyO*	**Dr. Banerjee, University of California Los Angelos**
	S^{54} *cn bw sp/CyO*	"
	S^{11N23} *cn bw sp/CyO*	"
	$W^{1}S^{1hs}$*/CyO*	"
(Star **heat shock transformant line)**	S^{hs8}*/CyO*	"
Star **(duplication)**	*DP(2;2)*S^{hs}*/CyO*	"

Star	***cn bw/SM6a,IKE 4.28.1***	**Dr. Zipursky, University of California Los Angelos**
	cn bw/SM6a, IV KE 2.4.2	
	cn bw/SM6a,VIII KE 4.1.1	
	VII 30E1,cn bw/SM6a	
	II 2.27 E.1, cn bw/SM6a	
	E3X12X, cn bw/sSM6a	
	cn bw/SM6a, VII KE.3.22	
	cn bw/SM6a, II KE 1.0.4	
	cn bw/SM6a, VIII KE 3.6.C	
	II 2.8.E1,cn bw/SM6a	
	cn bw/SM6a, 1X KE 4.0.1.	
	E3R12X, cn bw/SM5	
	E3R34X, cn bw/SM5	
	E3RIIX, cn bw/SM5	
rugose	rg^1/rg^1	**Bloomington Stock Center**

***sevenup* (enhancer trap line)**	***svp/TM3***	"
***rhomboid* (enhancer trap line)**	***rho*^{x81}**	**Ethan Bier UCSD Sandiego California**
***Scabrous*(enhancer trap line)**	**E^{72}**	**Nick Baker Albert Einshtien College of Medicine**

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